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Functional and biochemical analysis of acidic amino acid transport and utilization by *Pseudomonas putida* KT2440

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This thesis is dedicated to my Parents
as a token of gratitude

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1. Introduction

1.1 Background of the project

Pseudomonas putida is a Gram-negative γ-proteobacterium. As a saprophytic organism it is found in soil and water, but also associated with plant roots. This kind of beneficial interaction with plants includes the genus *Pseudomonas* among the plant-growth-promoting rhizobacteria (PGPR). Some pseudomonads like *P. aeruginosa* and *P. syringae* are pathogenic, introducing new challenges in human health and plant disease control. Beneficial pseudomonads, on other hand, are useful for the degradation of biogenic and xenobiotic pollutants, for controlling bio-agents in certain plant diseases and for biocatalytic/biotransformation processes (Nelson *et al.* 2002 and Lee & Cooksey 2000). The members of the genus *Pseudomonas* are extremely versatile organisms, showing a wide range of adaptability to biotic and abiotic stresses. This is partly due to their large genomes (6-6.3 Mbp as compared to 4.6 Mbp in *E. coli*) that encode a great variety of transporters, enzymes, and regulatory systems to cope with altered environmental conditions (http://www.tigr.org/).

The *Pseudomonas putida*-plant interaction in the rhizosphere is largely based on root exudates which function as rich nutrient medium for the growth of root-associated bacteria while some constituents promote bacterial adherence and mediate communication between guest and host (de Weger *et al.* 1995). Generally, amino acids, monosaccharides, organic acids are considered the major exudate constituents, but other components like vitamins and putriscine are also present (Fan *et al.* 1997). The predominant amino acids in root exudates are the acidic amino acids Asp and Glu and their amides Asn and Gln. In barley-root exudates, for instance, the acidic amino acids and their amides account for almost 50% of all amino acids (Barber & Gunn 1974). Jones and Darrah showed that in corn rhizosphere, amino acids afford as much as 220 mg nitrogen/kg of soil dry weight, while nitrate and ammonia together account for less than 60 mg/kg (Jones & Darrah 1993). In addition to their nutritional value, some amino acids also play a role in the plant-*Pseudomonas* interaction. So Vilchez *et al* showed that certain proline-catabolizing genes of *P. fluorescens* are strongly induced by root exudates (Vilchez *et al.* 2000). However, the roles of amino acids in the plant-bacterial interaction are still poorly characterized.

Our particular interest in the utilization of acidic amino acids is due to the astonishing ability of many pseudomonads to grow at maximum rates on these amino acids as the sole source of

carbon and nitrogen. In previous studies, Sonawane *et al.* showed that growth of *P. putida* in acidic amino acids induces a group of genes which help bacterium to utilize amino acids effectively (Sonawane *et al.* 2003b). Among these genes there is a previously uncharacterized ABC transporter as well as a two-component system *aauR/S* (for acidic amino acid utilization) that controls the expression of σ^{54}-dependent genes. The major goals of this study were to characterize the role of these systems in acidic amino acid uptake and utilization by *Pseudomonas putida*.

1.2 Choice of organism

Pseudomonas putida KT2440, a derivative of the soil isolate mt-2, first described by (Franklin *et al.* 1981) was selected for this study. This strain is a non-pathogenic, efficient root colonizer. It is found in the rhizosphere at high cell densities, where it controls plant diseases and enhance crop yields. In addition, strain KT2440 has attractive features like the ability to degrade certain xenobiotics and polychlorinated biphenyl pollutants, and hence is used in bioremediation. Moreover, *P. putida* KT2440 is a popular model organism for metabolic and genetic studies and suitable for laboratory experiments without any special security measurements. It grows well on a variety of standard carbon and nitrogen sources and shares many features of other *Pseudomonas spp.* like *P. aeruginosa* and *P. syringae*. The complete annotated genome of *P. putida* KT2440 is available along with 6 other genomes of different *Pseudomonas spp.* which greatly helps in the analysis and manipulation of gene sequences of this organism.

1.3 Bacterial amino acid metabolism: an overview

Nitrogen is present in many cellular metabolites and commonly assimilated either from inorganic or organic sources. Inorganic nitrogen is assimilated by reduction into ammonia, however, organic nitrogen is assimilated by many pathways depending on the organism. Variety of nitrogenous compounds are utilized by microorganisms to meet their nitrogen requirements. These compounds range from simple dinitrogen (N_2) or nitrate (NO_3^-) to complex organic compounds like amino acids and nucleotides. Many bacteria, including the group of enteric bacteria prefer ammonium ions (NH_4^+) as a source of nitrogen. In absence of ammonia, the alternate nitrogen sources are catabolized via diverse metabolic pathways.

Amino acids are important precursors for biosynthetic processes and can be metabolically divided into two groups. The first group is termed glucogenic. These amino acids yield α-keto acids when deaminated thus feed major metabolic pathways like the TCA cycle and gluconeogenesis. Glucogenic amino acids are efficient alternative carbon sources for bacteria when glucose is not available. The second group belongs to ketogenic amino acids, which yield acetyl-coA or acetoacetate after degradation and thus are unable to provide precursors for gluconeogenesis. The metabolic pathways of amino acids can be categorized into two classes: degradative pathways used for amino acid utilization and biosynthetic pathways for production of other nitrogen-containing compounds.

The regulation of these pathways is controlled by the coordinate expression of genes, depending on the intracellular availability of nitrogenous compounds. Nitrogen limitation inside the cells affects expression of around 100 genes in *E. coli* by the process involved, is known as nitrogen response (Ntr response). Ntr response is also known in many microorganisms which controls the utilization of alternative nitrogen sources. Amino acids are building blocks for proteins and precursor compounds for important metabolites like vitamins, lipids, carbohydrates, nucleotides, and many secondary metabolites. In most of these pathways Glutamate (Glu) and glutamine (Gln) serve as key nitrogen donors. In *E. coli* aspartic acid and serine are the biggest donor of Carbon atoms for biosynthetic processes and Glu, Gln are the major nitrogen donors (Reitzer 2003).

1.4 Ammonia assimilation by enteric bacteria

The growth of microorganisms is frequently limited by the availability of nitrogenous compounds. In enteric bacteria, the assimilation of ammonium ions (NH_4^+) is the major route for nitrogen utilization. The pathways involved can be divided into two groups i) assimilation of nitrogenous precursors and ii) production of new nitrogen-containing compounds. The specificity of the nitrogen assimilation metabolic pathways may vary from organism to organism, but in most of the cases glutamate (Glu) and glutamine (Gln) serve as key intermediates. In enteric bacteria, the ammonia assimilation pathway basically involves the GDH and GS/GOGAT pathways (see Fig. 1.1, Tyler 1978).

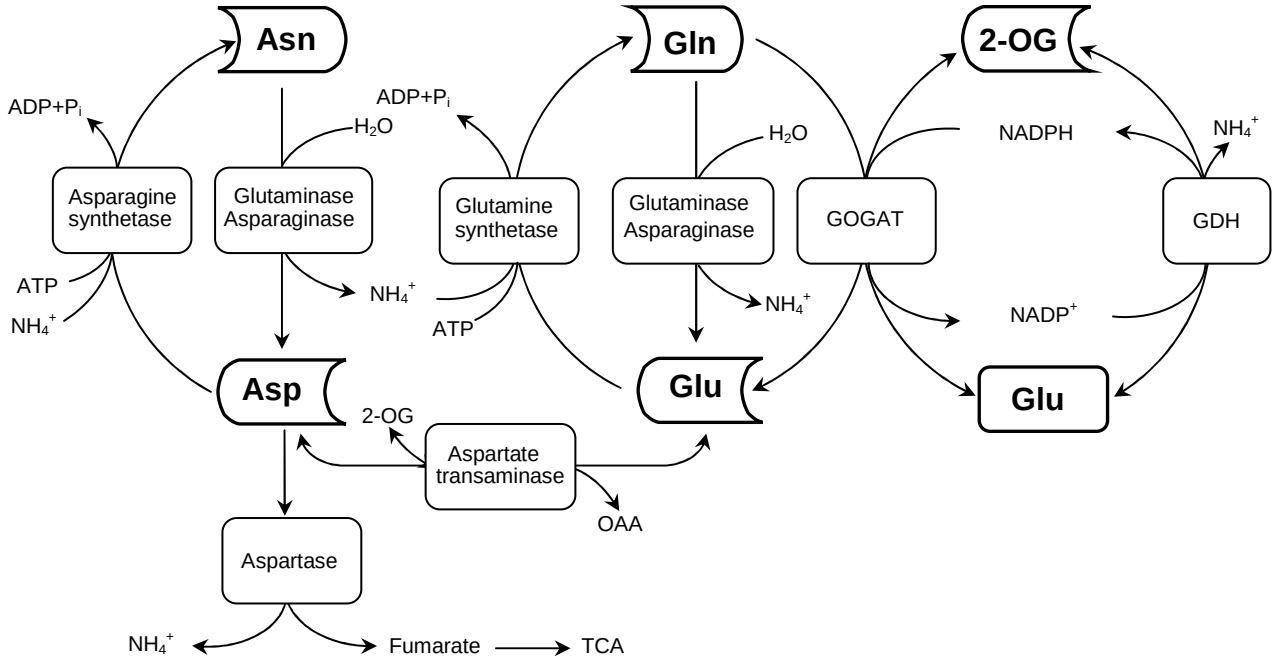


Figure 1.1: Ammonia assimilation pathways of enteric bacteria.

1.4.1 GDH pathway

This pathway involves the enzyme glutamate dehydrogenase (EC 1.4.1.4) which catalyzes the reductive synthesis of glutamate from 2-oxoglutarate and ammonia using NADPH as the coenzyme. In most of the microorganisms, GDH has relatively high K_m values for 2-oxoglutarate and ammonia (~1 mM), which suggests that it is the preferred pathway when intracellular ammonium concentrations are high, while this pathway is ineffective under ammonia-limited conditions.

$$\text{2-Oxoglutarate} + \text{NADPH} + H^+ + NH_3 \longrightarrow \text{Glutamate} + NADP^+$$

1.4.2 GS/GOGAT pathway

This is an ubiquitous and very important pathway of ammonia assimilation. It involves glutamine synthetase (glutamate-ammonia ligase, GS) and glutamate synthase (glutamine-2-oxoglutarate aminotransferase, GOGAT). GS catalyzes the ATP-dependent synthesis of glutamine from ammonia and glutamate. Its K_m for ammonia (~0.1 mM) is 10 times lower than that of GDH.

$$\text{Glutamate} + NH_3 + \text{ATP} \longrightarrow \text{Glutamine} + \text{ADP} + P_i$$

$$\text{2-Oxoglutarate} + \text{Glutamine} + \text{NADPH} \longrightarrow 2\ (\text{Glutamate}) + NADP^+$$

The available evidence indicates that GS is a highly regulated enzyme at both the transcriptional and the post-translational levels. In Gram-negative bacteria, GS is reversibly modified by a bifunctional enzyme adenylyltransferase (ATase) in response to nitrogen availability. Adenylylation and deadenylylation regulate the catalytic activity of GS. When intracellular level of nitrogen is sufficient, ATase catalyzes the transfer of AMP from ATP to the subunits of GS which progressively inactivates the enzyme. Conversely, when the intracellular nitrogen level is low, the adenylyl group is removed from GS and the enzyme becomes active again. In *E. coli*, two protein components, P_I and P_{II}, are involved in the adenylylation and deadenylylation process. P_I contains an adenyltransferase whose ability to adenylylate or deadenylylate GS is specified by the P_{II} protein and by the concentrations of P_I, ATP, UTP, Gln and 2-ketoglutarate (Merrick & Edwards 1995). Structural analyses of GOGAT revealed that the enzyme is a heterodimer, whose larger glutaminase subunits and smaller transaminase subunits are encoded by genes *gltB* and *gltD* respectively. GOGAT is essential for the de-repression of the Ntr response in many bacteria as it removes Gln which represses the Ntr system.

1.5 Enzymes of amino acid utilization

In ammonia-limited conditions, cells utilize many alternative nitrogen sources such as nitrate, urea and amino acids (Magasanik 1993). As already mentioned, *E. coli* and other enterobacteria derive all their nitrogen from Glu or Gln (Reitzer 2003). Nevertheless, Glu and Gln are inferior to NH_4^+ in supporting growth of enteric bacteria. In *Pseudomonas* the situation is different. Several strains of *P. fluorescens* and *P. putida* rapidly grow on acidic amino acids and their amides, even when these are supplied as the sole source of carbon and nitrogen (Hüser *et al.* 1999 and Sonawane *et al.* 2003a).

1.5.1 Asparagine synthetase

The reactions catalyzed by asparagine synthetase use either Gln or ammonia as a nitrogen source to convert Asp to Asn. Two families of asparagine synthetases have been found. Members of the AsnA family which occur in *E. coli* and *Klebsiella aerogenes* use only ammonia as the amino group donor (Humbert & Simoni 1980 and Reitzer & Magasanik 1982). The other group is the

AsnB family, members of which were found in both prokaryotes and eukaryotes (Hughes *et al.* 1997 and Scofield *et al.* 1990). These enzymes use both Gln and ammonia as the nitrogen donor, but Gln is the preferred one.

1.5.2 Glutaminase/Asparaginase

Enzymes that catalyze the hydrolysis of Gln and Asn are widely distributed in microorganisms. One such group of amidohydrolases, called glutaminase/asparaginase, convert asparagine or glutamine to their respective dicarboxylates, aspartate and glutamate. According to their sub-cellular localization and kinetic properties, there are two major subgroups (Class I and Class II). Class I asparaginases are constitutive cytoplasmic enzymes with a marked preference for L-Asn. By contrast, class II enzymes, encoded by the *ansB* gene, are located in the periplasm and show a wider specificity for L-Asn and L-Gln as well as for their D-isomers (Cedar & Schwartz 1967 and Derst *et al.* 2000). The role of asparaginases has been studied extensively in Gram-negative bacteria such as *E. coli* (Cedar & Schwartz 1967), *Salmonella enterica* (Jennings & Beacham 1993), *Erwinia chrysanthemi* (Gilbert *et al.* 1986), *Vibrio proteus* (Sinha *et al.* 1991) and also in some Gram-positive organisms such as *Bacillus subtilis* (Atkinson & Fisher 1991) and *Staphylococcus aureus* (Rozalska & Mikucki 1992).

In *Pseudomonas*, the acidic amino acids (Asp, Glu) and their amides (Asn, Gln) strongly and specifically induce the periplasmic glutaminase/asparaginase isoenzyme (Klöppner, 1999 and Hüser *et al.* 1999). However, the physiological roles of class II glutaminases/ asparaginases and their regulation in *P. putida* KT2440 at the molecular level are not well understood.

1.5.3 Aspartase

This enzyme is also referred to as aspartate ammonia lyase. It plays an important role in amino acid metabolism by reversibly converting the product of glutaminase/asparaginase, L-Asp, to fumarate and ammonium ion. Thus, it feeds the carbon skeleton of Asp into the TCA cycle.

1.5.4 Aspartase transaminase

This enzyme is also known as aspartate aminotransferase or glutamate-oxaloacetate transaminase, catalyzes the reversible formation of oxaloacetic acid and Glu from Asp and 2- oxoglutarate.

1.6 Molecular control of nitrogen metabolism in bacteria

As already mentioned, enteric bacteria can utilize various organic and inorganic nitrogenous compounds like amino acids, nucleotides, peptides, N_2 and NO_2 etc, but ammonia is a preferable natural nitrogen source. Glutamate provides 80% of the nitrogen for molecular synthesis inside cells while 15% of nitrogen comes from the amide group of glutamine (Magasanik 1993). The nitrogen regulation in enteric bacteria is largely controlled by the global nitrogen regulatory system (Ntr). Ammonia availability can suppress the expression of genes required for alternate source utilization. Growth in a minimal medium with a single organic nitrogen source is slower than that with ammonia, loss of a few metabolic enzymes in bacteria can prevent the utilization of a variety of nitrogen sources (Reitzer 2003).

1.6.1 Global nitrogen regulatory system (Ntr)

The Ntr system of enteric bacteria consists of four proteins: 1) Uridyl transferase/Uridyl removing enzyme, 2) P_{II}; a glutamine synthetase (GS) regulating protein, 3) NtrB and 4) NtrC, the NtrB/C are parts of a two component system. The Ntr system has been extensively studied in *E. coli* (Jiang *et al.* 1998 and Atkinson & Ninfa 1998). In this system the first sensing protein of nitrogen level is UT/UR which is a bifunctional enzyme regulating uridinylation of the P_{II} protein and ultimately starting the Ntr response cascade. When the cells are nitrogen limited, i.e. when the [Gln]/[2-OG] ratio is low, this stimulates UTase to transfer UMP to protein P_{II}, which then activates glutamine synthetase. When nitrogen supply is sufficient, the high [Gln]/[2-OG] ratio facilitates deuridinylation of the P_{II} protein by UR which, in turn reduces the activity of GS (Fig. 1.2). Glutamine plays a central role in this nitrogen control system by regulating the uridinylation of P_{II} (Jiang *et al.* 1998). The regulation of GS by adenylation and deadenylation is discussed in section 1.4.2.

NtrC (NRI- Nitrogen regulator I, the product of *glnG* or *ntrC*) and NtrB (NRII - Nitrogen regulator II, the product of *glnL* or *ntrB*) constitute a prototypical two-component system (TCS) where NtrB is the sensor histidine kinase while NtrC is the cognate response regulator. In the absence of P_{II}, NtrB act as protein kinase, it autophosphorylates and transfers the phosphate group to the receiver domain of NtrC. Phosphorylated NtrC then induces σ^{54} dependent expression of

several genes. Binding of free PII to NtrB stimulates its phosphatase activity which dephosphorylates NtrC. Thus P_{II} acts as a regulatory switch of this system (see Fig. 1.2).

In *E. coli* the Ntr response controls many operons which are actively involved in nitrogen metabolism like the *gltIJKL* operon for glutamate uptake, *glnHPQ* for glutamine metabolism, *astCADBE* for arginine catabolism, *hisJMPQ* for histidine transport, *codBA* for cytosine transport, *yhdWXYZ* for polar amino acid transport, *glnALG* and *gln-amtB* for ammonia transport and *argT* for basic amino acid transport (Zimmer *et al.* 2000).

It is still unclear whether the Ntr system also operates in pseudomonads. However, some knock-out models related to the nitrogen regulation have been studied. Mutations in the nitrate reductase genes *nasB* and *gltB* of *P. putida* KT2442 abolish the ability to utilizate nitrogen sources like amino acids, urea or low levels of ammonium (Eberl *et al.* 2000). *P. aeruginosa* PA14 mutants lacking the *rpoN* gene (*ntrA*, *glnF*) were unable to grow on nitrate, glutamate and histidine as sole source of carbon and nitrogen and were non-motile (Hendrickson *et al.* 2001). A study by Sonawane *et al,* showed that in *P. putida* KT2440 the GOGAT encoding *gltB* gene is essential for utilization of acidic amino acids and regulation of several key enzymes of nitrogen regulation (Sonawane & Rohm 2004).

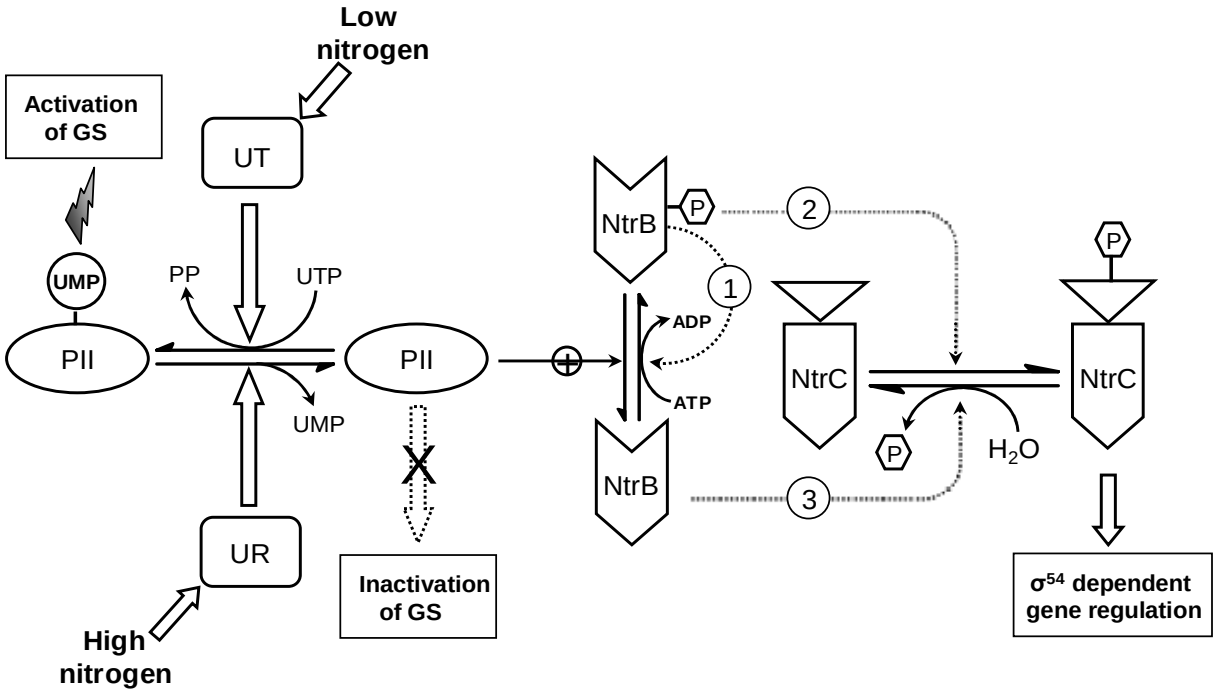


Figure 1.2: Nitrogen control pathway by PII protein and Ntr system of *E. coli.* The reactions (1); autophosphorylation of NtrB, (2) phosphotransfer to NtrC, (3) dephosphorylation (see text).

1.7 Two-component systems

1.7.1 Introduction

Rapid adaptation to environmental challenges is essential for bacterial survival. The change in temperature, pH of environment, osmolarity and nutrient availability or biotic factors like inter-species competition or host availability, alters the population and growth behavior of microorganisms. These altered factors in the broad sense are termed as stress which is managed by bacterial cells through genetic and biochemical adaptations. The first type of system for adaptation phenomenon is sensing system, which is widely accomplished by protein molecules (sensor) present in bacterial membranes. After sensing the multitude of signals, the sensor induces response at genetic and physiological levels by modifying the function of macromolecules. These adaptive responses in most of the bacteria are orchestrated by a class of regulatory systems known as two-component systems.

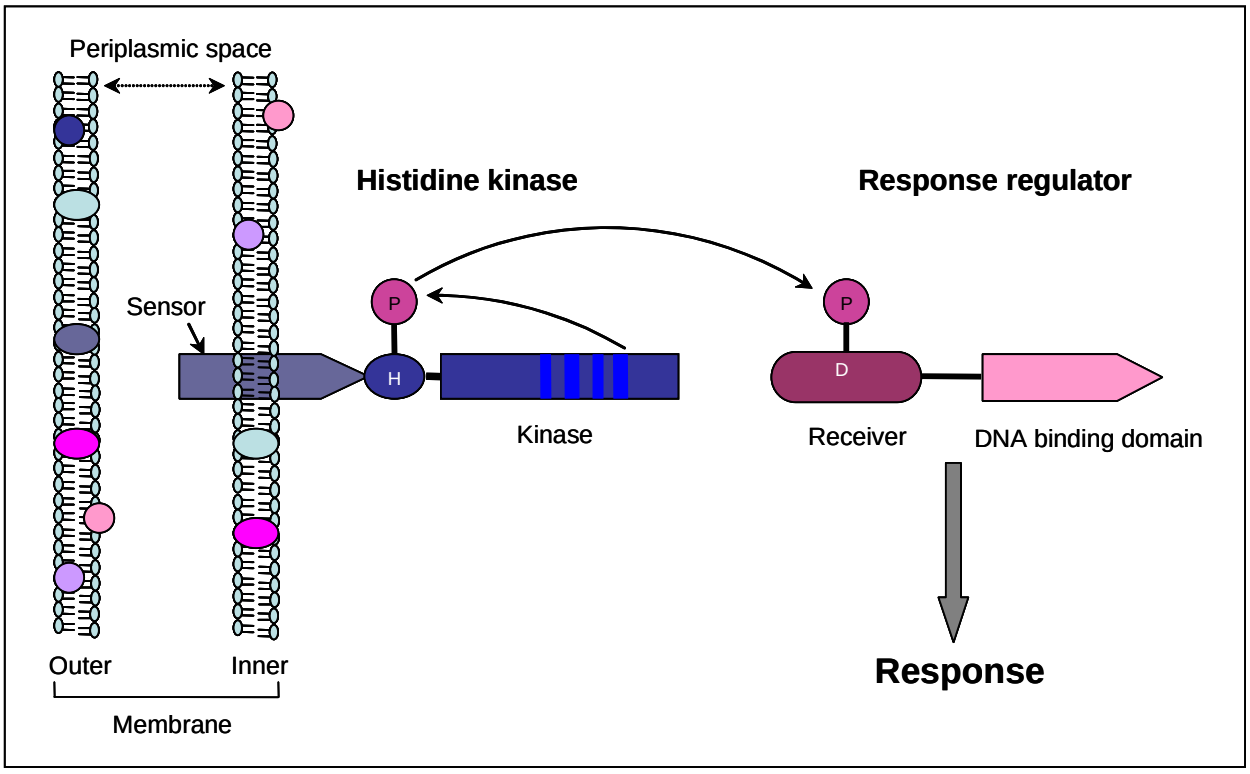


Figure 1.3: A prototypical two-component system with histidine kinase (Sensor kinase) and response regulator with their mode of function.

The structure and functions of different two-component systems have been described in several recent reviews (Stock *et al.* 2000, Foussard *et al.* 2001 and West & Stock 2001). These systems are distributed over all groups of organisms (archaea, bacteria and some eukaryotes). However, their abundance in each group differs substantially. Genome sequencing of many microorganisms revealed an uneven distribution of two-component systems in their genomes, e.g. 0 in *Mycoplasma*, 29 in *E. coli*, 35 in *Mycobacterium tuberculosis*, 38 in *Synechocystis*, 3 in *Candida albicans*, 2 in *Arabidopsis thaliana* and none is reported in *Homo sapiens* and *Drosophila melanogaster* (Koretke *et al.* 2000).

1.7.2 Mechanism of action

A typical two-component system consists of a membrane bound sensor protein (histidine kinase) and a cytoplasmic regulator protein (response rgulator). The histidine kinase senses the stimuli, autophosphorylates at one of its conserved histidine residues, creating a high energy phosphoryl group which is subsequently transferred to the aspartate residue of response regulator protein. The phosphorylation-induced conformational changes of response regulator protein cause the response by interactions with other protein molecules or by the direct control of transcription mechanisms. This histidyl-aspartyl phosphorelay basically involves three phosphotransfer and two phospho-protein intermediates:

1. Autophosphorylation: HK-His + ATP $\longleftrightarrow$ HK-His~P + ADP
2. Phosphotransfer : HK-His~P + RR-Asp $\longleftrightarrow$ HK-His + RR-Asp~P
3. Dephosphorylation : RR-Asp~P + H_2O $\longleftrightarrow$ RR-Asp + P_i

Most two-component systems work according to the above mentioned scheme, but some of them function by multiple phosphorelay steps. These are called hybrid two-component systems. For instance, the chemotaxis control system in *E. coli* involves a cytoplasmic sensor, CheA, which autophosphorylates and then transfers the high energy phosphoryl group to CheY and CheB. The sporulation control system of *B. subtilis* has two sensor proteins (KinA and KinB), which autophosphorylate in stress conditions and transfer phosphate group first to Spo0F and then to Spo0B, that ultimately phosphorylates the Spo0A response regulator (Errington 1993).

1.7.3 Domain structure of two-component systems

1.7.3.1 Histidine kinases

The histidine kinases can be divided into 2 classes (see figure 1.4). Class I kinases are membrane-coupled proteins that exhibit three types of basic structural arrangements.

i) Simple kinases (Fig. 1.4a) occur in many typical two-component systems. They consist of a transmembrane receiver protein coupled to a kinase domain, with a conserved histidine residue in it. Sensing of the cognate stimulus leads to autophosphorylation of this histidine, which then transfers the phosphoryl residue to the response regulator (e.g. the EnvZ /OmpR system of *E. coli*.)

ii) In one type of hybrid kinase (Fig. 1.4b), the histidine kinase domain is associated with two further domains, a receiver domain that accepts the primary high-energy phosphate from the HK domain at a conserved aspartate residue. The phosphate is then transferred to a conserved His in the so-called Hpt domain from where it is eventually transferred to the response regulator (e. g. ArcB/ArcA system of *E. coli* for anoxic redox control, BarA/UvrY for carbon metabolism control in *E. coli,* BvgS/BvgA in *Bordetella pertussis* controlling virulence).

iii) In a second type of hybrid kinase (Fig. 1.4c), the Hpt domain is not linked to the receiver but functions independently as cytoplasmic proteins (e. g. KinA-KinB-Spo0F-Spo0B/Spo0A controlling sporulation *Bacillus subtilis*.)

Class II histidine kinases (Fig. 1.4d) have a cytoplasmic sensor that is coupled to a membrane receptor and show an entirely different architecture with the HPt domain at the N-terminus. They are mainly involved in chemotaxis e. g. CheA/CheB of *E. coli* (Taylor & Zhulin 1999).

In class I kinases the kinase domain is linked to the transmembrane sensor protein by a linker region ranging from 40-180 amino acids. These linkers have coiled-coil structure and play an important role in transmitting the signal to the kinase core (Park & Inouye 1997). The kinase core is around 350 amino acid in length and is responsible for ATP binding and directing transphosphorylation. A central feature is a conserved histidine residue in the so-called ‘H box’ that accepts phosphate from the kinase (Fig. 1.5a). The kinase domain has several conserved boxes (N, G1, F and G2) in its binding cleft (Fig. 1.5b). In most histidine kinases, the H box is part of a

dimerization domain at the N terminus of the kinase domain. In some HKs it is a separate protein or coupled to the C terminus of kinase domain and is then termed Hpt domain, e.g. in ArcB and CheA of *E. coli*. The N, G1, F, G2 boxes are largely conserved, while the spacing between these boxes somewhat varies in different HKs. The folding pattern of the kinases differs from those of Ser/Thr/Tyr kinases but closely resembles with ATPases, Gyrase b, MutL and Hsp90 families of proteins.

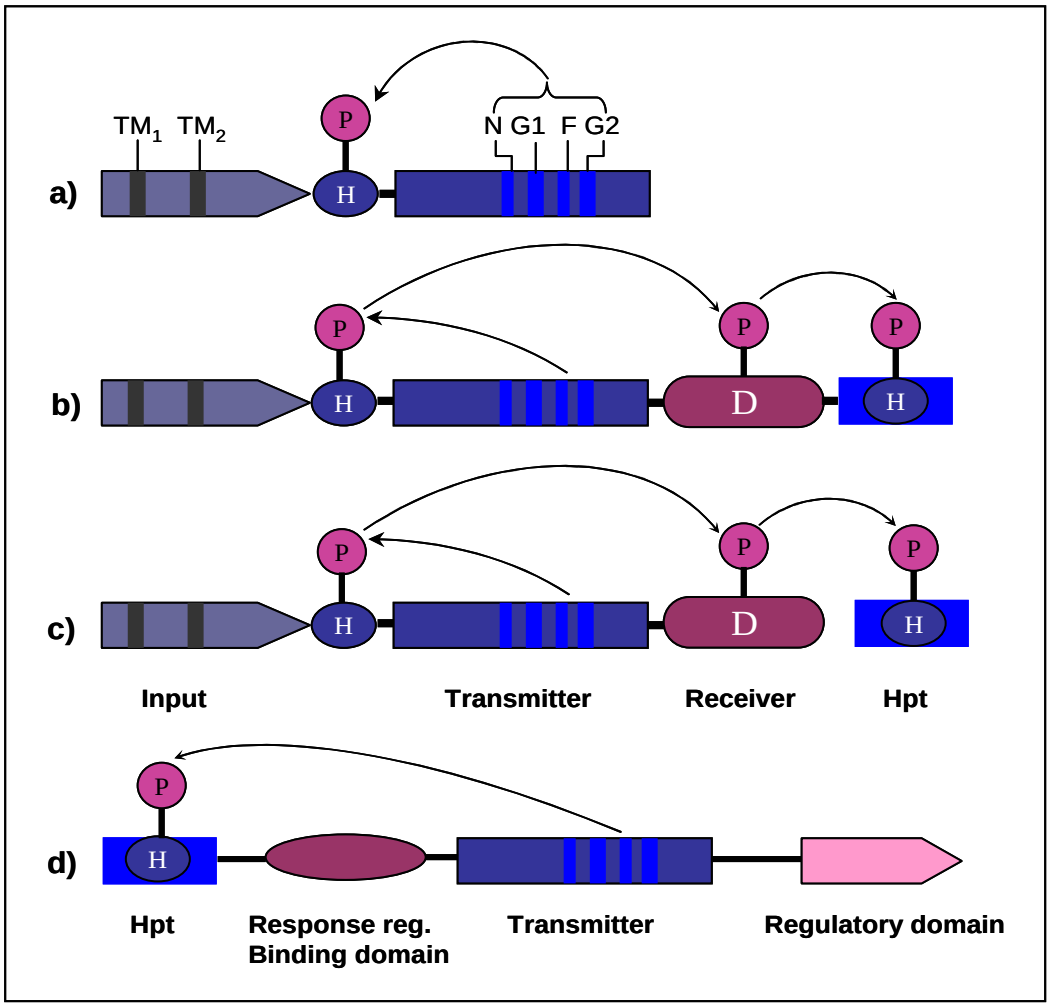
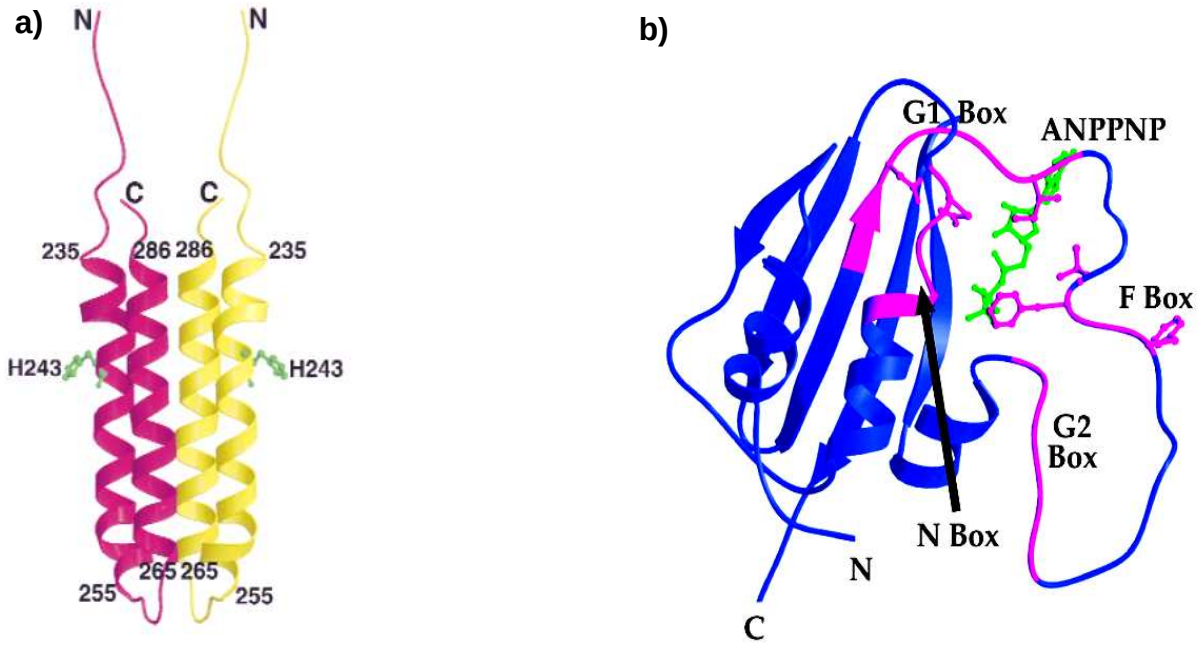


Figure 1.4: Modular organization of the histidine kinases. In class I, (a, b, c) the N-terminal input domains are variable in length senses variety of signals, they are mostly membrane bound. The C-terminal transmitter or kinase domain has highly conserved histidine residue with N, G1, F and G2 boxes. Hybrid kinases (b, c) contain a receiver and Hpt domain which may be associated with HK or cytoplasmic. Class II histidine kinases are involved in chemotaxis coupled to membrane receptor with Hpt domain at N-terminal and regulatory domain at C-terminal. These are normally cytoplasmic but associated with membrane coupled receptor for functional regulation.



Form (Tomomori *et al.* 1999) From (Stock *et al.* 2000)

Figure 1.5 Structure of Histidine kinase. **(a)** Dimerization domain (DHp), NMR structure of the homodimeric core (residues 223–289) of EnvZ. His_{243} is the site of autophosphorylation and phosphate transfer reactions. The structure comprises a four-helix bundle formed by two identical helix-turn-helix subunits, revealing the molecular assembly of two active sites within the dimeric kinase. **(b)** Ribbon diagram of kinase catalytic core (CA) of EnvZ protein of *E. coli* AMP-PNP molecule is shown in ball and stick bound at active site, the nitrogenous base buried deep inside and phosphate group facing outside, easily accessible to histidine residue of DHp/Hpt domain. The α-helices and β-sheets are arranged in β1-α1-β2-α2-β3-β5-β4 topology. N, G1, F and G2 boxes are present in loops.

The solution NMR structure of the dimerization domain (DHp) of EnvZ has 4 helix bundles arranged in antiparallel manner (Fig. 1.5a). H_{243} is conserved in both dimers and stimulates trans-autophosphorylation. The DHp domain of EnvZ represents the structural arrangement in the majority of all known HKs (Tomomori *et al.* 1999). Two-component systems containing a Hpt domain are frequently found in hybrid two-component systems like CheA of *E. coli*, ArcB of *E. coli* and Spo0B of *B. subtilis*. The Hpt domain does not exhibit kinase or phosphatase activity but is involved in cross-communication between different proteins (Bilwes *et al.* 1999).

1.7.3.2 Response regulators

Most response regulator proteins consist of two domains: a conserved N-terminal regulatory phosphate-accepting domain and a variable effector domain. Most of the response regulators are transcription factors, i.e. they have DNA-binding effector domain. Some regulators contain diverse domains with specific functions. The σ^{54}-binding response regulators contain an additional ATPase domain (Fig. 1.6d). Some regulators even lack an effector domain and just function as communication modules between two proteins (Fig 1.6a).

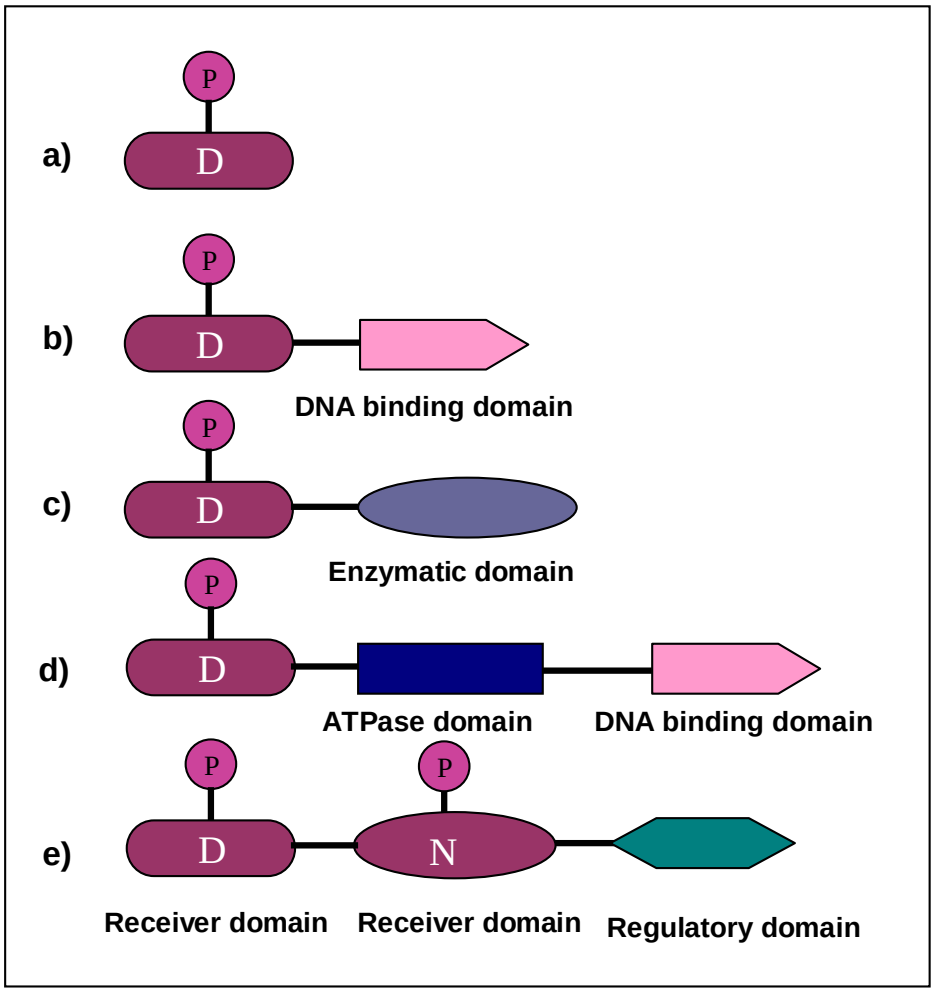


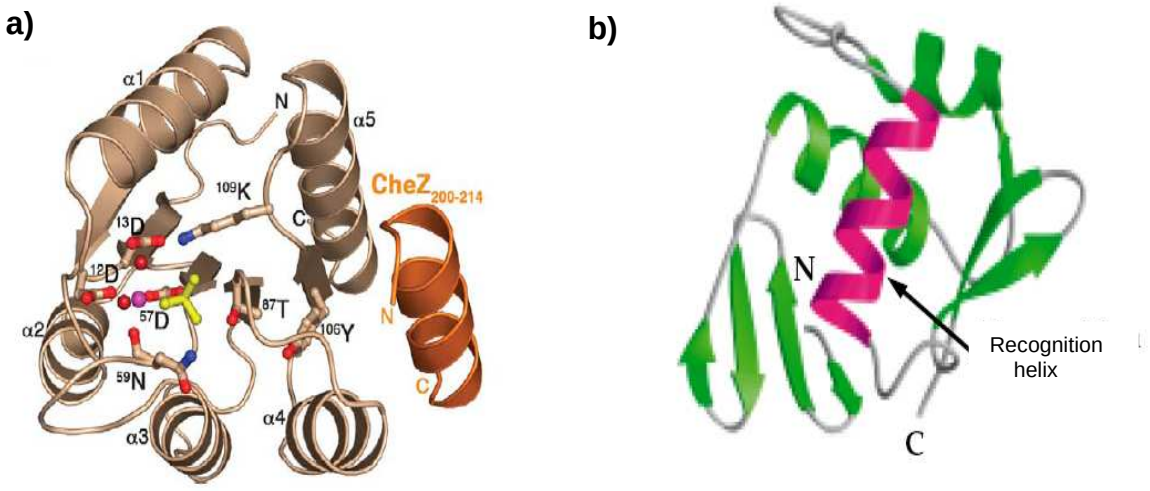
Figure 1.6: Modular organization of response regulators. These proteins share a common N-terminal receiver domain which is phosphorylated at highly conserved aspartate residue. Response regulators may carry different other domains which may contain DNA binding H-T-H motif like in b and c, Other domains may be ATPase domain, σ^{54} interacting domain, reciever with conserved asparagines for phosphorylation, other enzymatic or regulatory domains. Normally receiver domain in response regulators regulates the function of other coupled domains.

The receiver domains of most response regulators contain a conserved acidic cluster which is involved in the coordination of Mg^{2+}, a cofactor required for phosphorylation and dephosphorylation. The majority of response regulators (e.g. 25 out of 32 in *E. coli*) act as DNA binding transcription factors (Mizuno 1997). Some RRs have a C-terminal functional enzymatic domain e.g. chemotaxis protein (CheB) of *E. coli.* The DNA binding domains can be divided into three major families-

i) OmpR: proteins of this family are the largest and best-characterized, contain a recognition helix interacting with the major groove of DNA and flanking loops or wings for binding to the minor grooves (Harlocker *et al.* 1995).

ii) NarL: These factors, which regulate genes involved in nitrate and nitrite metabolism contain a helix-turn-helix (HTH) motif for binding.

iv) NtrC: Members of this family of response regulators control the σ^{54}-dependent transcription of genes or operons involved in nitrogen metabolism.



From (Guhaniyogi *et al.* 2006)

From (Martinez-Hackert & Stock 1997)

Figure 1.7: structure of response regulator domains. **(a)** CheY of *S. typhimurium*, contains a cluster of conserved acidic residues (side chain and Cα atoms shown and labelled) that bind Mg^{++} and form the active site for phosphoryl transfer. D57 is phosphate acceptor. The central core has 5 β-sheets surrounded by 5 α-helices. **(b)** The effector C-terminal domain of *E. coli* OmpR, the recognition helix shown in magenta *colour* and DNA binding winged-helix domain in *green colour.*

The chemotaxis protein CheY serves as a basic model of a regulatory domain. This protein is a doubly wound 128 residue α/β protein with a central five-stranded parallel β-sheet surrounded by 5 α-helices. Asp_{57}, the site of phosphorylation is located in solvent-exposed loop (Fig. 1.7a). Other highly conserved residues (Asp_{12}, Asp_{13}, Thr_{87}, and Lys_{109} surround the active site of the regulatory domain (Volz & Matsumura 1991). The helix-turn-helix motif (Fig. 1.7b) binds with major grooves in target DNA.

1.8 Sigma factors in bacterial gene expression

Sigma factors are a group of proteins involved in initiating gene expression by binding to RNA polymerase. The σ-RNAP complex (holoenzyme), guided by the sigma factor, then recognizes the promoter region that marks the transcription start site. Sigma factors also contribute to transcription by facilitating DNA strand separation, which must occur before RNA polymerase can begin copying the DNA code. Once transcription starts, the sigma factor disengages from the RNA polymerase, becoming available for complex formation with different RNA polymerases.

Sigma factors can be structurally classified into two distinct families. The first one is the σ^{70} family which involves the "housekeeping" sigma factor σ^{70} and stress response sigma factors, which can be divided into three subclasses σ^{B}, σ^{S}, and σ^{F}. These latter factors enhance cell survival under different stress conditions and maintain virulence. The general stress factor σ^{B} controls the transcription of more than 100 genes under different stress conditions in *S. aureus* (Giachino *et al.* 2001) and plays an important role in *Listeria monocytogenes* during early infection of the intestinal epithelium (Kim *et al.* 2004). σ^{S} plays a key role in protecting *E. coli*, *S. enterica* and *P. aeruginosa* from different environmental stress conditions, including starvation, hyperosmolarity, oxidative damage, and reduced pH. Other sigma factors control sporulation, flagella formation and the heat shock response. (Fang *et al.* 1992 and Gruber & Gross 2003). Within the σ^{70} family there is a large, phylogenetically distinct subfamily called the extracytoplasmic function (ECF) factors. These sigma factors are responsible for regulating a wide range of functions, all involved in sensing and reacting to conditions in the membrane, periplasm, or extracellular environment.

1.8.1 σ^{54} factors and nitrogen metabolism

A second family of sigma factors is called as σ^{54} (σ^{N}, RpoN). These factors occur in many different bacteria and control metabolism under nitrogen-limited conditions. The number of sigma factors in bacterial genome is highly variable, but most genomes contain only one copy of the σ^{54} gene or none at all. The *P. fluorescens* Pf5 genome is the largest one sequenced (7 Mbp), and it also has by far the largest number of sigma factors (4 σ^{70}-family factors and 28 ECF sigma factors) but only a single σ^{54}.

A number of findings indicate that, nitrogen assimilation in pseudomonads is under direct control of σ^{54}: So *rpoN* mutants were unable to utilize nitrate, urea, alanine, glycine, isoleucine, leucine and serine as nitrogen sources, in addition this mutation affected the utilization C_4-dicarboxylates (Köhler *et al.* 1989). σ^{54} also regulates other genes like alginate synthesis and flagellar genes needed for cellular infection and attachment of *P. aeruginosa* (Boucher *et al.* 2000 and Dasgupta *et al.* 2003). It is also involved in virulence control of *P. syringae* and *Vibrio cholerae* and in other human and plant pathogens (Zhao *et al.* 1999 and Chatterjee *et al.* 2002).

1.8.2 Mechanism of σ^{54} transcriptional regulation

σ^{54} regulated genes have a binding motif (GC/GG) in their promoter upstream sequences located at -12/-24 relative to the transcription start site and an additional enhancer sequence, usually situated between -100 to -150 (Carmona *et al.* 1997).

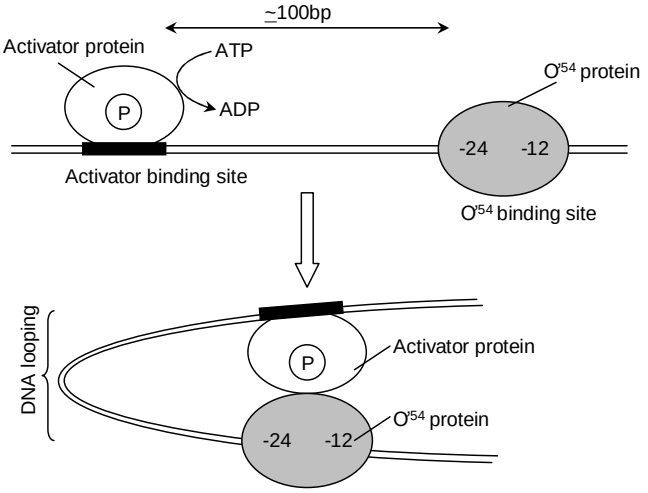


Figure 1.8: Role of activator proteins in the transcription of σ^{54}-dependent genes (see text)

The activator proteins of σ^{54} genes usually contain a receiver domain, a DNA binding domain of the helix-turn-helix type and a σ^{54} recognition domain. They bind to DNA when phosphorylated at a specific site in their receiver domain (see section 1.7.2). Activators of this type interact with DNA and σ^{54} at the same time to introduce a bend in DNA, ultimately forming an open promoter complex as a prerequisite for transcription (Carmona *et al.* 1997).

1.9 Transport of nitrogenous compounds in bacteria

As already mentioned, nitrogenous compounds are essential precursors for the synthesis of many cellular macromolecules. Microorganisms can adapt to the changing availability of nitrogen in the biosphere by expressing a number of different uptake systems.

1.9.1 Ammonium transport

Ammonium transport in prokaryotes is controlled by an ubiquitous family of proteins of the ammonium transport family AMT (Thomas *et al.* 2000). The Amt system in enteric bacteria is regulated by global nitrogen regulatory (NtrB/NtrC) two-component system (Jayakumar *et al.* 1986 and Arcondeguy *et al.* 2001). The *amt* gene of *E. coli* is transcriptionally linked to a second gene *glnK*, which encodes a signal transduction protein belonging to the P_{II} family. When external NH_4^+ is limiting, Amt facilitates the transport of ammonium into the cell, where it is converted to glutamine by glutamine synthetase. When the intracellular Gln pool is replenished, the GlnK protein is deuridylated and binds to Amt which, in turn, inhibits ammonium transport (Williams & Miller 2001).

1.9.2 Nitrate transport

Many bacteria reduce nitrate to nitrite which is subsequently converted to ammonia. Transport of nitrate is facilitated by two different means, i) *narK* family transporters and ii) ABC transporters (Moir & Wood 2001). Nitrate uptake and utilization are mainly controlled by a P_{II} like protein that modulates the global nitrogen control system. G*lnB* mutants of *B. subtilis* and *R. leguminosarum* failed to utilize nitrate as sole source of nitrogen, a finding emphasizing the essential role of this system in nitrate utilization and uptake (Amar *et al.* 1994).

1.9.3 Amino acid transporters

Most amino acids are transported by ABC transporters, which are widely distributed in all prokaryotic and eukaryotic organisms. The specificity of these transporters with respect to the solute may be broad or very narrow. On the basis of sequence homology, amino acid transporters of the ABC type can be categorized as follows: i) General amino acid transporters, ii) Branched chain amino acid transporters, iii) Basic amino acid transporters, iv) acidic amino acid transporters. Other systems for amino acid transportation, especially Glu and Asp also occurs by secondary transporters known as Na^+ of H^+ dependent symporters. This kind of symporters use membrane potential or pH gradient, acts as driving force for Glu/Asp uptake. The anionic Glu transport is electrogenic, which at least involves co-transport of two protons or equivalents (de Vrij *et al.* 1989 and Tolner *et al.* 1995a). ABC transporters are more active and common for amino acid uptake in bacteria.

1.10 ABC transporters

ATP **B**inding **C**assette (ABC) transporters couple ATP hydrolysis to the uptake of solutes across the membrane. In Gram-negative bacteria, ABC transporters are made up of four-five different domains or subunits; the extra-cytoplasmic solute-binding protein (SBP) binds the solute specifically, membrane spanning domains (MSDs), function as channels for solute transport, while cytoplasmic nucleotide-binding domains (NBDs) interact with the cytoplasmic surface of the MSDs to supply energy by ATP hydrolysis (Fig. 1.9). In Gram-negative organisms all the domains are separate and interact together but in Gram-positive organisms, the solute binding domain is not separate and coupled with transmembrane domains. ABC transporters constitute one of the largest superfamilies of proteins and are widely distributed over archaea, bacteria and eukaryotic cells. So, for instance, there are 47 ABC transporters in humans and 69 in *E. coli*. ABC transporters are predominantly involved in the uptake of sugars, amino acids, metals, ions, and many other solutes. They also participate in exporting bacterial toxins and several harmful metabolites and antibiotics from cytoplasmic pool, thus providing antibiotic resistance to organism (Davidson & Chen 2004).

The ABC family is currently subdivided into 22 subfamilies of prokaryotic importers, 24 subfamilies of prokaryotic exporters and 10 subfamilies of eukaryotic transporters (Quentin & Fichant 2000). A detailed classification is available in the ABC transporter data base (http://www-

biology.ucsd.edu/~msaier/transport/). The grouping of ABC transporters is largely based on the phylogenecity, substrate specificity, their functional assignment and evolutionary criteria (Saier 2000). Although sequence similarity between different families of MSDs is usually 25-30% or less, the existence of highly conserved residues in these domains indicates that they use similar mechanisms for coupling of transport to ATP hydrolysis.

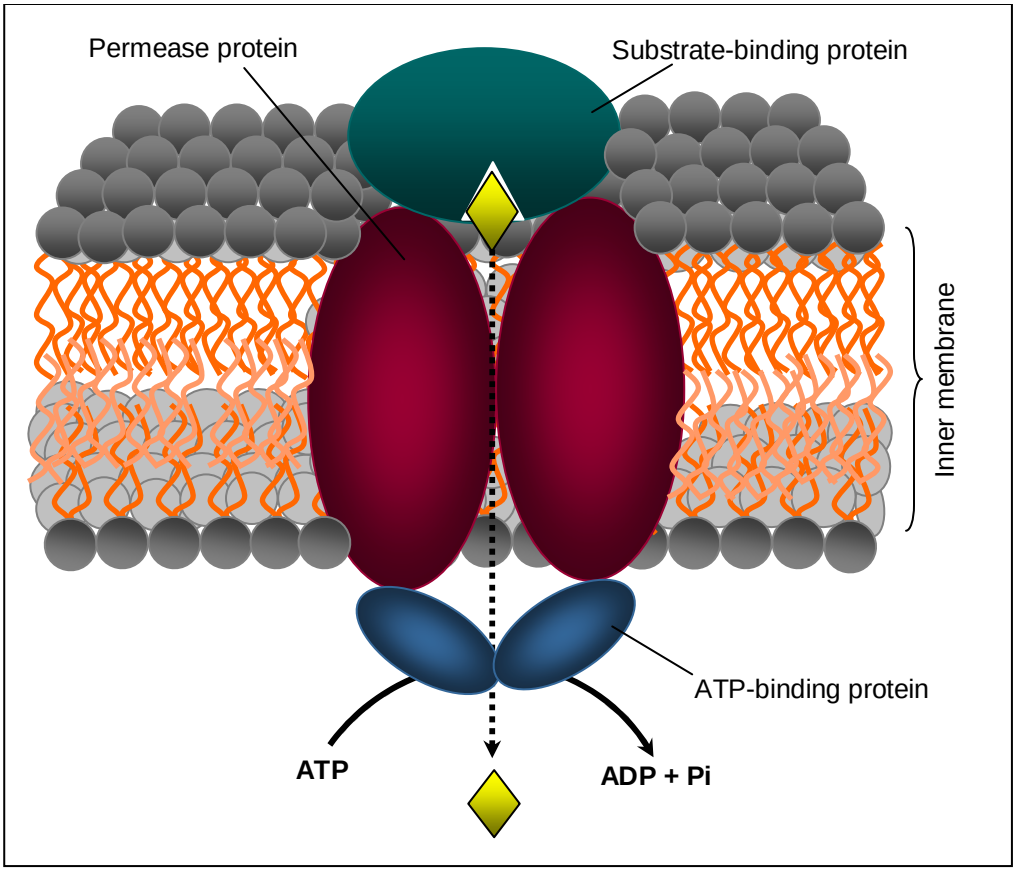


Figure 1.9 Scheme of a typical prokaryotic ABC transporter made up from a substrate-binding protein (SBP), two membrane-spanning domain (MSDs) and two nucleotide-binding domains (NBDs). This type of ABC transporters are commonly found in Gram-negative bacteria.

1.10.1 Structure of ABC transporters

Solute binding proteins (SBP) associated with ABC transporters exhibit three types of folding pattern all of them consisting of two lobes with the solute binding site positioned between them. Class I and II binding proteins include glucose, maltose and galactose binding proteins. Upon ligand binding they undergo conformational changes involving the bending of a hinge that joins both lobes which promotes the closing of the lobes around the ligand (Quiocho & Ledvina 1996).

The conformational changes and bending of lobes is directly correlated with substrate specificity as substrate analogues that cannot induce cleft closure are not transported (Ledvina *et al.* 1998). Class III binding proteins include iron-siderophore binding proteins and vitamine B_{12} receptors. These proteins have a relatively rigid hinge and do not undergo substrate-induced conformational changes (Clarke *et al.* 2000).

Well-characterized permeases are the MSDs HisQ and HisM of histidine ABC transporters from *S. typhimurium* and *E. coli* (Baichwal *et al.* 1992 and Liu *et al.,* 1997). The N-terminal part of the permeases interacts with the substrates and contains some conserved residues which are used to classify the ABC transporters (Davidson & Chen 2004). The nucleotide-binding domains also contain highly conserved sequences, such as the Walker A and B motifs, a LSGGQ motif, and the so-called Q loop. These motifs are present in nearly all NBDs suggesting that they use a common functional mechanism (Ramaen *et al.* 2003). The proper function of transporters depends on the interaction of the solute-binding protein with the MSDs. *In vitro* studies with a reconstituted HisQM complex showed that ATP hydrolysis by the nucleotide-binding domain HisP requires both HisJ and HisQM (Liu & Ames 1998).

1.10.2 Mechanism of transport

In Gram-negative organisms small solutes can diffuse through the outer membrane via porin proteins while larger molecules like vitamin B_{12} and iron-siderophore complexes need active transport. The periplasmic solute-binding protein has two roles in transport as judged by the properties of SBP knock-out mutants. Binding protein mutants of the *E. coli* maltose transporter were still able to transport maltose, although with greatly increased K_d (100-200 μM as compared to 1-2 μM in the wild type (Treptow & Shuman 1985 and Merino *et al.* 1995). This suggests that the binding proteins are responsible for high- affinity transport which is the characteristic of this kind of transporters. Secondly, the solute binding protein activates ATP hydrolysis by transmitting a signal through the transmembrane permease subunits and thus triggers transport (Davidson & Chen 2004 and Merino *et al.* 1995).

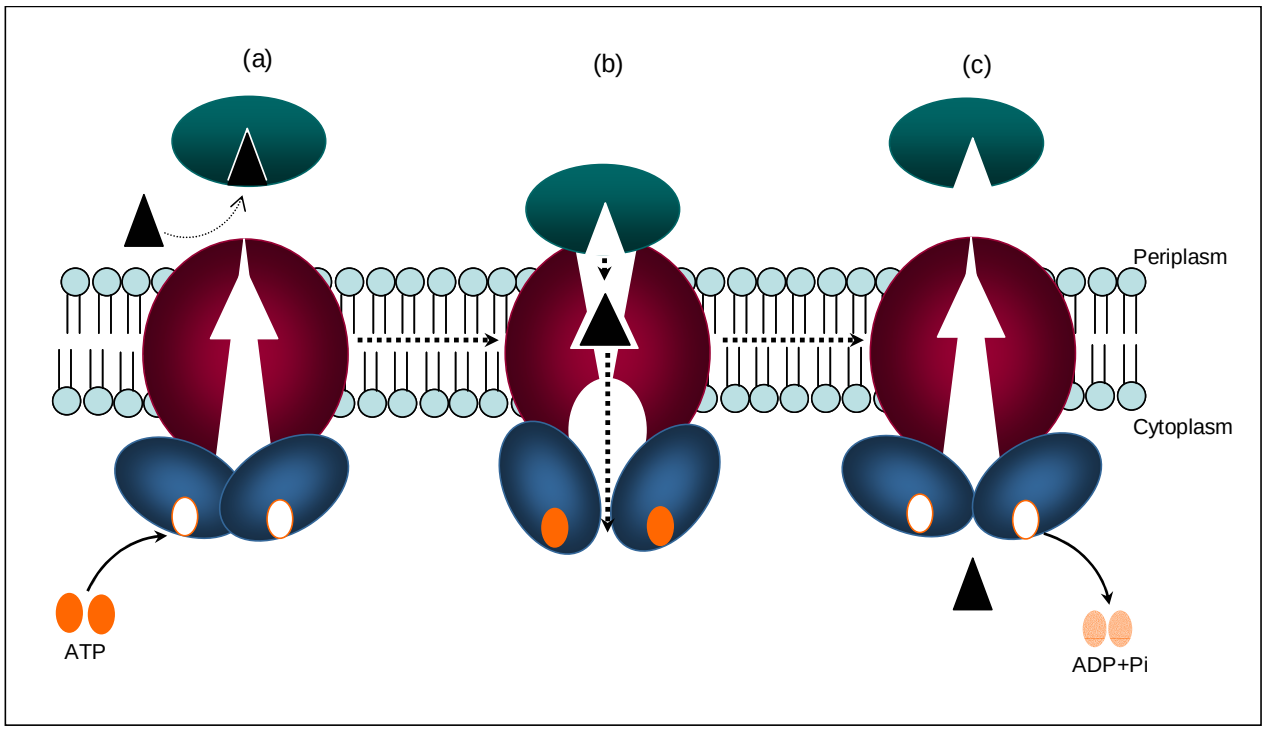


Figure 1.10: Model for maltose transport by the maltose ABC transporter **(a)** The loaded maltose-binding protein (MBP) in its closed conformation interacts with the permease, simultaneously inducing ATP hydrolysis and starting transport. **(b)** Presumed transition state of ATP hydrolysis. The MBP is still tightly bound with the permease but in an open conformation. **(c)** After ATP hydrolysis the solute is translocated and the transporter returns to its original conformation. The MBP activates the ATPase activity by bringing the two NBDs in close proximity, so that a complete nucleotide binding site at the dimer interface can form (according to Chen *et al.* 2001).

1.10.3 Amino acid ABC transporters in pseudomonads

Pseudomonads genome encodes many ABC transporters. The functional assignment of most of them is still based on sequence similarity alone. The *Pseudomonas putida* KT2440 genome codes for 117 ABC transporters which is 32.1% fraction of total transporters, in contrast *E. coli* encodes only 74 ABC transporters, accounts for 20% total transporters (http://www.membranetransport.org/). According to their functional roles they can be divided into 5 groups i) amino acids, peptide and amine transporters, ii) anion transporters, iii) cation and iron transporters, iv) carbohydrate, alcohol and organic acid transporters, v) putative ABC transporters with unknown function. In *P. putida* 20 different systems are assigned as amino acid ABC

transporters, some of them with specificity for cysteine, proline/glycine betain, branched-chain amino acids, basic amino acids and aromatic amino acids. However, most of them are putative and general amino acid transporters without defined specificity.

1.11 Aims and objectives of this study

The major aims of this study were-

- A better understanding of the control of acidic amino acid metabolism in *P. putida* KT2440 at the genetic and biochemical level.
- Characterization of the functional role of the AauRS two-component system.
- Functional characterization of the acidic amino acid ABC transporter AatJMQP.
- Structural and functional studies of the Glu/Asp binding protein AatJ.

2. Materials

2.1 Microorganisms and plasmids

Stock of microorganisms were prepared in sterile 40% glycerol. Routinely used stocks were stored at -20°C whereas, longer used stocks were stored at -80°C.

Strain or plasmid	Genotype/origin/ sequence	Reference
***P. putida* strains**		
KT2440	*mt*-2, *hsd*R1 ($r^- m^+$)	(Bagdasarian *et al*. 1982)
KT*aauR*	*aauR*$^-$ derivative of KT2440	(Sonawane A., 2003c)
KT*aauS*	*aauS*$^-$ derivative of KT2440	(Sonawane A., 2003c)
KT*aatP*	*aatP*$^-$ derivative of KT2440	This work
KT*aatJ*	*aatJ*$^-$ derivative of KT2440	This work
***E. coli* strains**		
Top10	F$^-$, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *recA*1, *araD*139, Δ*(ara-leu*)7687, *gal*U, *gal*K, *rps*L, *end*A1, *nup*G	(Invitrogen)
BL21	F$^-$, *omp*T, *hsd*S ($r_B^- m_B^-$), *gal*, *dcm*	(Amersham biosciences)
HB101	*sup*E44, *hsd*S20 ($r_B^- m_B^-$), *rec*A13, *ara*-14, *pro*A21, *ac*Y1, *gal*K2, *rps*L20	(Amersham Biosciences)
Plasmids		
pUC19	Cloning vector, *amp*^{r}	
pK18	Cloning vector, *kan*^{r}	(Pridmore, 1987)
pTrcHisA	Expression vector, pBR322 *ori*, *lac*I^q, p*trc*, *lac*O, *amp*^{r}	(Invitrogen)
pFLAG-ATS	Expression vector, *omp*A, p*tac*, *lac*I, *amp*^{r}	(Sigma)

2.2 Antibiotics

Antibiotics were prepared as stock solutions, filter sterilized and stored at -20^{0}C.

Antibiotics	Stock (mg/ml)	Final concentration (µg/ml)
Ampicillin	100	100
Kanamycin	40	40
Tetracycline	10	25
Chloramphenicol	100	50

2.3 Oligonucleotide Primers

Oligonucleotide primers were synthesized commercially by MWG BioTech, Ebersberg, Germany. The sequencing of vectors were also performed by MWG BioTech.

2.3.1 Primers used for protein over-expression

Primer names	Sequence	Restriction site
aauRFor	5'-CGCggatccATGAACCAAGCGCCTCTTAC-3'	BamHI
aauRRev	5'-CCGgaattcTCAGGCGAGGCCGTATTTTTTC-3'	EcoRI
aauSFor	5'-CGCggatccATGCGGCGCAAAGTGATCG-3'	BamHI
aauSRev	5'-CCGgaattcTCATGGATGCTCTGCCGATTCG-3'	EcoRI
aatJFor	5'-CGCaagcttGAGGAGCTCACCGGCACCCT-3'	HindIII
aatJRev	5'-CCCgaattcTCAGGACTTCTTCTCGTCCGCAG-3'	EcoRI

2.3.2 Primers used for Knockout of *P. putida* KT2440 genes

Primer names	Sequence	Restriction site
PP1068_For	5'-CGCggatccTCGGGCAA**G**GTCCACCCT-3'	BamHI
PP1068_Rev	5'-CCCaagcttTACACGGTCGAGCAGCGCCA-3'	HindIII
PP1071_For	5'-CGCggatccTCGCATGAC**A**ATCCAGCTGGC-3'	BamHI
PP1071_Rev	5'-CCCaagcttGTCAACACGTTCTTGCCGGCC-3'	HindIII
pK18_For	5'-CAATTTCACACAGGAAACAGCTATGACC-3'	-
PP1068_361	5'-CTTGGTGGCTTCCGCCTCGCT-3'	-
PP1071_414	5'-GTCTTTGTATGCCGGCTGACCGT-3'	-

2.3.3 Primers used for amplification of promoters

Primer names	Sequence	Restriction site
PromFor_PP1071	5'-CCCggatccCGCATCGAGCCTTTCCGGTTGGGC-3'	BamHI
PromRev_PP1071	5'-CCCaggcttCTCCGGGGTTCGCCAGGCCGAAT-3'	HindIII
PromRev_117	5'-GCTCGTTCGGTTATCCGAACGCATGGGC-3'	-
PromRev_136	5'-CGCATGGGCTGCCAACCTATCCCGG-3'	-
PromRev_157	5'-CCGGTATCGCTTGCGGTAACTATGCAGTGC-3'	-
PromRev_202	5'-ACGTAGTGAGCTTTGCTCTTTGCCTGGAGAG-3'	-
PromFor_2453	5'-ACCAACTATGTTAACAGGACCGTTGTTTG-3'	-
PromRev_2453	5'-CGACAATGTGTGATGTTGCACCGA-3'	-

2.3.4 Primers used for site directed mutagenesis of *aatJ*

Primer names	Sequence
R71A_For	5'-GGTCACCTCGCAAACC**GC**TATTCCGCTGGTGCAG -3'
R71A_Rev	5'-CTGCACCAGCGGAATA**GC**GGTTTGCGAGGTGACC -3'
S86A_For	5'-ACCTGGAGTGTGGC**G**CCACCACCAACAAC -3'
S86A_Rev	5'-GTTGTTGGTGGTGG**C**GCCACACTCCAGGT -3'
T87A_For	5'-CTGGAGTGTGGCTCC**G**CCACCAACAACGTC -3'
T87A_Rev	5'-GACGTTGTTGGTGG**C**GGAGCCACACTCCAG -3'
T88A_For	5'-GAGTGTGGCTCCACC**G**CCAACAACGTCGAG -3'
T88A_Rev	5'-CTCGACGTTGTTGG**C**GGTGGAGCCACACTC -3'
R93A_For	5'-ACCAACAACGTCGAG**GC**CCAGCAACAGGTTGGCTTC -3'
R93A_Rev	5'-GAAGCCAACCTGTTGCTGG**GC**CTCGACGTTGTTGGT -3'
R174A_For	5'-GCTCGAAAGCGGC**GC**CGCCGTGGCCTT -3'
R174A_Rev	5'-GAAGGCCACGGCG**GC**GCCGCTTTCGAGC -3'
S86A/T87A_For	5'-ACCTGGAGTGTGGC**G**CC**G**CCACCAACAACGTC -3'
S86A/T87A_Rev	5'-GACGTTGTTGGTGG**C**GG**C**GCCACACTCCAGGT -3'
T87A/T88A_For	5'-TGGAGTGTGGCTCC**G**CC**G**CCAACAACGTCGAG -3'
T87A/T88A_Rev	5'-CTCGACGTTGTTGG**C**GG**C**GGAGCCACACTCCA -3'

2.4 DNA and protein ladders

Name	Company name
MassRuler™ DNA (ladder low range)	Fermentas SM0388, St.Leon-Rot
MassRuler™ DNA (ladder high range)	Fermentas SM0393, St.Leon-Rot
GeneRuler™ DNA (100 bp ladder)	Fermentas SM0241, St.Leon-Rot
O´GeneRuler™ DNA (100 bp ladder)	Fermentas SM1148, St.Leon-Rot
PageRuler™ (protein ladder)	Fermentas SM0661, St.Leon-Rot
Roti-Mark 10-150 (SDS-PAGE mearker)	Roth Life science T850.1, Karlsruhe
Dalton Mark VII	Sigma (SDS7), Steinheim

2.5 Enzymes

Enzyme name	Company name
Taq-Polymerase	Fermentas, St.Leon-Rot
PfuTurbo-Polymerase	Stratagene Heidelburg
Restriction enzymes	Roche Boehringer, Mannheim
Alkaline phosphatase (CIP)	Fermentas St.Leon-Rot
T4-DNA ligase	Fermentas St.Leon-Rot
T4- Polynuceotide kinase (PNK)	Stratagene Heidelburg
DNase I	Fermentas, St.Leon-Rot
DpnI	Fermentas, St.Leon-Rot
Enterokinase Tag-off™	Novagen, Darmstadt

2.6 Radioactive labeled chemicals

Chemical name	Company name
[γ-P^{33}] ATP	Hartmann Analytic, Braunschweig
[^{14}C(U)] L-Aspartic acid	Hartmann Analytic, Braunschweig
[^{14}C(U)] L-Glutamic acid	Hartmann Analytic, Braunschweig

2.7 Kits

Kit name	Company name
Bacterial genomic DNA isolation kit	Sigma, Steinheim, Germany
Bichniconinic acid protein estimation (BCA) kit	Pierce, Rockford, IL, USA
Plasmid isolation Mini Genelute™	Sigma, Steinheim, Germany
QIAquick PCR purification kit	Qiagen GmbH, Hilden, Germany
QIAquick Gel Extraction kit	Qiagen GmbH, Hilden, Germany
Tag-off™ Enterokinase kit	Novagen, Darmstadt, Germany
Gel-shift assay kit	Molecular Probes, Leiden, Netherlands

2.8 Chemicals

Chemical name	Company name
Acrylamide, Bis acrylamide (30:0.8)	Roth, Karlsruhe
Acrylamide, Bis acrylamide (19:1)	Roth, Karlsruhe
Agarose	Sigma, Steinheim
Agar	Fluka, Buchs
Amino acids (Analytical grade)	Sigma, Fluka and Merck
Ammoniumpersulfate	Merck, Dermstadt
ATP (Adenosine 5' triphosphate)	Sigma-Aldrich, Steinheim
ADP (Adenosine 5' diphosphate)	Sigma-Aldrich, Steinheim
α-ketoglutarate	Sigma, St Louis MO
Bromophenol Blue	Sigma-Aldrich, Steinheim
ß-Mercaptoethanol	Merck, Dermstadt
Bovine serum albumin (BSA)	Roth, Karlsruhe
Coomassie Brillant blue	Serva, Heidelburg
Dithiothreitol (DTT)	Roth, Karlsruhe
Ethedium bromide	Roth, Karlsruhe
EDTA	Sigma-Aldrich, Steinheim
D (+) Glucose	Merck, Dermstadt
Glycerol	Sigma-Aldrich, Seelze
Glycine	Sigma-Aldrich, Seelze
Imidazole	Fluka, Buchs
HEPES	Fluka, Buchs
L-AHA (L-aspartic acid ß-hydroxymate)	Sigma, St Louis MO

Chemical name	Company name
Luria-Broth	Invitrogen
N,N,N`,N`-Tetramethylendiamine (TEMED)	Roth, Karlsruhe
NADH (β-nicotinamide adenine dinucleotide)	Fluka
O-phthalaldehyde	Serva, Heidelburg
Piperazine	Fluka, Buchs
Phosphoenol pyruvate (PEP)	Sigma, St Louis MO
Q-Sepharose™ Fast flow	GE Healthcare Biosciences, Uppsala
Sephacryl S-100	GE Healthcare Biosciences, Uppsala
Saturated phenol	Roth, Karlsruhe
Sodium dodecylsulphate	Merck, Dermstadt
Serva Blue G	Serva, Heidelburg
Salmon sperm DNA	Sigma-Aldrich, Buchs
Sucrose	Roth, Karlsruhe
Tris-base	Roth, Karlsruhe
Urea	Roth, Karlsruhe
Xylene cyanol FF	Sigma-Aldrich, Steinheim

2.9 Instruments

Apparatus	Model	Manufacturer
Autoclave	2540ELV, 5075ELV	Tuttnauer Systec, Wettenberg
Analytical Balance	R160P	Sartorius Research, France
Agarose gel electrophoresis	Easy cast™ models B1A and B2	Owl Scientific inc, Portsmouth, NH
Columns	Glass columns (manual packing)	Pharmacia Biotech, Uppsala, Sweden
	His-Trap™ FF	GE healthcare, Uppsala, Sweden
	MonoQ® (anion exchange)	Pharmacia Biotech, Uppsala, Sweden
	Superdex™ 200 (Gel filtration)	Pharmacia Biotech, Uppsala, Sweden
	Microspin™ PSR-300	GE healthcare, Uppsala, Sweden
	LichroSphere® 60RP Select B	Phenomenex, USA
Centrifuges	Biofuge pico, Biofuge fresco	Heraeus, Hanau, Germany
	Minifuge RF	Heraeus Sepatech, Hanau, Germany

Apparatus	Model	Manufacturer
	J2-21	Beckman, USA
CD-polarimeter	J-810	JASCO, UK
DNA Sequencing Unit	Sequi-Gen®	Bio-Rad, Hercules, USA
Electroporator	2510	Eppendorf, Hamburg, Germany
Freezers	-20, -80 (Environ Scan™)	Leibherr, Comfort Forma
FPLC	AKTA prime Waters 650 SE	Amersham Biosciences, Sweden Waters Milford, USA
Fraction collector	LKB Frac-100	Pharmacia Biotech, Sweden
Gel documentation	P91W, P91E	Mitsubishi, Japan
Heating block	Thermostat 5320	Eppendorf, Hamburg
HPLC	D-7500	Hitachi, Japan
Incubator Incubator shaker	Bacterial growth incubator CH4103	Heraeus, Hanau, Germany Infors, Bottmingen, Germany
Laminar hood	HERA safe	Heraeus, Hanau, germany
Microplate reader	EL_x808	Biotech instrument inc. USA
Micropipettes	0.5-2µl, 1-10 µl, 20-200µl, 100-1000 µl	Gilson, France
Phosphor Imager	STORM® with ImageQuant® image analysis software	Molecular Dynamics Sunnyvale, CA, USA
Phosphor Screens	20x25 cm and 35x43 cm (Kodak)	Amersham Biosciences, Sweden
Peristaltic pump	P1	Pharmacia Fine Chemicals, Sweden
PAGE units	Mini gels and large gels	Bio-Rad, Hercules, USA
pH meter	340	Mettler Toledo, Giessen
Power supplies	S200 and EPS 3501 XL, E865	Bio-Rad USA, Consort Sigma

2.10 Other disposable materials

Apparatus	Manufacturer
Membrane filter (0.2 μM)	Schleicher & Schull, Dassel
Membrane filter (0.45 μM)	Schleicher & Schull, Dassel
Millex®-GS (0.22 μM)	Mililipore, Bedford, USA
Microplates (96 well)	Greiner GmbH, Frickenhausen
DispoEquilibrium DIALYZER	Havard Apparatus, MA, USA

2.11 Computer programs and internet links

Programs	Links/company
Clustal W multiple alignment	http://www.ebi.ac.uk/Tools/clustalw/
NOMAD alignment	http://www.expasy.org/tools/nomad.html
BLAST	http://www.expasy.org/tools/blast/
Protein secondary structure prediction tool	http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html
Protein modeling server	http://swissmodel.expasy.org/
EasyPred3D modeling server	http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/
Aligner, complementer, Oligocalculator	http://www.justbio.com/aligner/index.php
Pseudomonas spp. Genome database	http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi
Promoter prediction tool (BPROM)	http://www.softberry.com
RCSB protein data bank	http://www.pdb.org/pdb/static.do?p=search/index.html
QuickChange primer design	http://www.stratagene.com/sdmdesigner/default.aspx
PromScan Promoter scanning	http://www.promscan.uklinux.net/RpoN/data.html
Fasta-Genome similarity search	http://www.ebi.ac.uk/fasta33/genomes.html
PyMol Viewer	DeLano Scientific LLC, California USA
DS visualizer 1.7	Accelrys Cambridge, UK
HyperChem	Hypercube, Inc.
Sigmaplot 8.0	Jandel Scientific
ProCite 5.0.3	Thomson ISI ResearchSof, Berkeley, CA, USA
Windows XP	Microsoft
Microsoft Office 2003	Microsoft

3. Methods

3.1 Safety

All bacterial inoculations and cultures were performed in safety hood under complete sterilized conditions. Media and solutions required for bacterial operations were autoclaved or filter sterilized before use. Bacterial waste was decontaminated by autoclaving for 30 min at 121°C. Ethidium bromide containing waste was stored in special containers labeled (carcinogenic) and processed properly before disposed off. Radioactive chemicals were used only under S1 security lab by monitoring dose exposure and waste disposal in labeled containers. Harmful chemicals were restrictedly used in fume hood. Safety gloves, glasses and apron were used in harmful and sterilized conditions.

3.2 Bacterial culture

3.2.1 Cultivation

Pseudomonas putida KT2440 and their mutants were grown in LB broth (Gibco BRL) or M9 minimal medium (Sambrook *et al,* 1989) or LB agar plates at 30ºC. *Escherichia coli* strains were grown on LB broth or LB agar at 37°C. The antibiotics ampicillin (100 μg/ml), Kannamycin (40 μg/ml), carbenicilline (100 μg/ml) were used for mutant selection and growth of plasmid harboring mutant strains. To examine the growth and transport of different amino acids in *P. putida,* cells were first grown on $M9^{+}$ medium supplemented with 20 mM ammonium chloride as nitrogen source and 22 mM glucose as carbon source for 15 hrs, collected by centrifugation at 4500 rpm, washed two times with M9 salt solution and then cells were resuspended into M9 medium containing; i) 5 mM amino acids as sole source of carbon and nitrogen or ii) 5 mM amino acid as nitrogen source and 22 mM glucose as carbon source. Bacterial growth was monitored by measuring the optical density of culture at 595 nm at regular time interval.

3.2.2 Storage

A loop-full of culture was streaked on LB plate and incubated overnight at required temperature. These plates were stored at 4ºC for 3 weeks for regular use. For longer storage, a single colony from streaked bacterial petri-dish was inoculated with 5 ml LB medium and grown overnight. This culture was mixed with 40% glycerol (v/v) in (1:1) ratio and stored at -80ºC. The culture was revived normally by inoculating 100 μl frozen culture into 5 ml growth medium.

3.3 Preparation and transformation of competent cells

3.3.1 Preparation of *E. coli* competent cells

A single colony of *E. coli* cells was grown in 5 ml LB medium overnight at 37ºC with 200 rpm shaking. 0.5 ml of this culture was re-inoculated with 50 ml fresh LB medium and grown until OD_{595} reached up to 0.5-0.6. The cells were transferred to sterile 50 ml polypropylene tube and recovered by centrifugation at 4000 rpm for 10 min at 4ºC. The medium was decanted off and traces of medium was drained out by inverting the tubes on sterile paper towel for 1 min. Pellet were resuspended in 20 ml of ice-cold $CaCl_2$ (0.1 M) by gentle vortexing and incubated at 4ºC for 96 min. The cells were harvested at 4000 rpm for 10 min at 4ºC and resuspended in 5 ml of 0.1 M cold $CaCl_2$. These cells were directly used for transformation or stored at -80ºC by addition of 1 ml 87% glycerol and dispensed as 100 µl aliquots.

3.3.2 Transformation of *E. coli* competent host cells

Frozen competent cells were thawed quickly and stored at 4ºC just before transformation. 10 µl ligated mixture or 25 ng vector was directly added to 100 µl competent cells and incubated in ice for 30 min. Heat shock was given for 1 min at 42ºC and chilled in ice for 5 min. 400 µl LB medium (without antibiotics) was added to transformed cells and incubated at 37ºC for 1 hr at 150 rpm shaking. The transformed cells were plated out on LB agar plates containing appropriate antibiotics and incubated at 37ºC.

3.3.3 Preparation of electro-competent *P. putida* KT2440 cells

A single colony from fresh plate of *P. putida* KT2440 was grown overnight in 5 ml LB medium at 30ºC with 200 rpm shaking. 0.5 ml culture of overnight grown cells was re-inoculated in 50 ml LB medium and grown until the OD_{595} reached to 0.5-0.6. The cells were stored on ice for 30 min and transferred to 50 ml sterile polypropylene tubes, harvested at 4000 rpm for 10 min at 4ºC. Cell pellets were washed two times with ice-cold 10% (v/v) glycerol and finally resuspended in 5 ml ice-cold 10% glycerol. The *P. putida* electro-competent cells were made fresh before use.

3.3.4 Electroporation of competent *P. putida* KT2440 cells

The vector DNA (200 to 400 ng) was added to freshly prepared 100 µl electro-competent cells in 1.5 ml eppendorf tube and stored on ice for 5 min. The DNA/cell mixture was loaded on chilled

electroporation cuvette (2 mm), without introducing any air bubble and tapped to insure that cells sit at the bottom. Moisture from outside of cuvette was dried by paper towel and placed in cuvette holder. A pulse of electricity at 2500 volt was delivered to the cells for 5 milli second and followed by immediate addition of 400 µl LB medium to the cuvette. Electroporated cells were transferred to 1.5 ml polypropylene tube and grown at 30ºC for 3 hr. The transformed cells were plated out on LB agar plates containing appropriate antibiotic and incubated at 30ºC.

3.4 Growth of *P. putida* KT2440 and mutants

3.4.1 Amino acids utilization study

Cells were first grown on $M9^{+}$ medium supplemented with 20 mM ammonium chloride as nitrogen source and 22 mM glucose as carbon source for 15 hrs, collected by centrifugation at 4500 rpm, 10 min at 4ºC and washed two times with M9 salt soluion, then the cells were resuspended into M9 medium containing i) 5 mM amino acids as sole source of carbon and nitrogen or ii) 5 mM amino acid as nitrogen source and 22 mM glucose as carbon source. 1 ml culture was taken out at specific time interval and centrifuged to collect the cells. The supernatant and cells were stored at -80ºC for amino acid quantification by HPLC analysis.

3.4.2 Amino acids uptake assay

The rate of Glu and Asp uptake were measured by quick filter assay method (Hosie *et al.* 2001). Single colonies from LB agar plates of *P. putida* KT2440, KT*aatJ* and KT*aatP* were grown overnight in 500 ml M9 medium supplemented with 20 mM NH_4Cl, 22 mM glucose and 40 µg/ml kannamycin in mutants. Cells were harvested at 4500 rpm for 10 min at 4ºC and washed two times with M9 salt solution, resuspended in 50 ml M9 salt solution and stored at 4°C (storage time should not exceed than 2 hrs). The cells were starved for 30 minutes at 30°C by gentle agitation at 150 rpm. The assay was initiated by addition of 5 µM (200 mci/mMol), ^{14}C labeled L-glutamate and L-aspartate (Hartmann Analytic GmbH, Germany) to 500 µl culture, 100 µl aliquots were taken out from culture at specific time intervals and added to 400 µl stop solution containing M9 salt solution with 50 mM same unlabelled amino acid. In competition experiments unlabelled amino acids were added before 2 minutes of labeled amino acid addition. The cultures were filtered through nitrocellulose membrane (0.45 µm pore size), washed two times with 500 µl stop solution and dried

at room temperature for 12 hrs, immersed in 6 ml scintillation cocktail and were counted on Beckman Coulter^{TM} LS6500 (USA) scintillation counter.

3.5 Survival of cells in amino acids

For survival studies of *P. putida* and their mutants in amino acids; cells were grown overnight in $M9^{+}$ medium, washed with M9 salt solution and resuspended in M9 medium containing amino acids as sole source of carbon and nitrogen. The viability count of cells was determined by serial dilution technique until 3-4 weeks. The protocol in brief, 100 µl culture was diluted serially up to 10^6 to 10^7 then, 100 µl of this culture was plated out in LB agar plate (with or without antibiotics). These experiments were done in triplicate. The plates were incubated at 30ºC for 24 hrs for growth and colonies were counted manually.

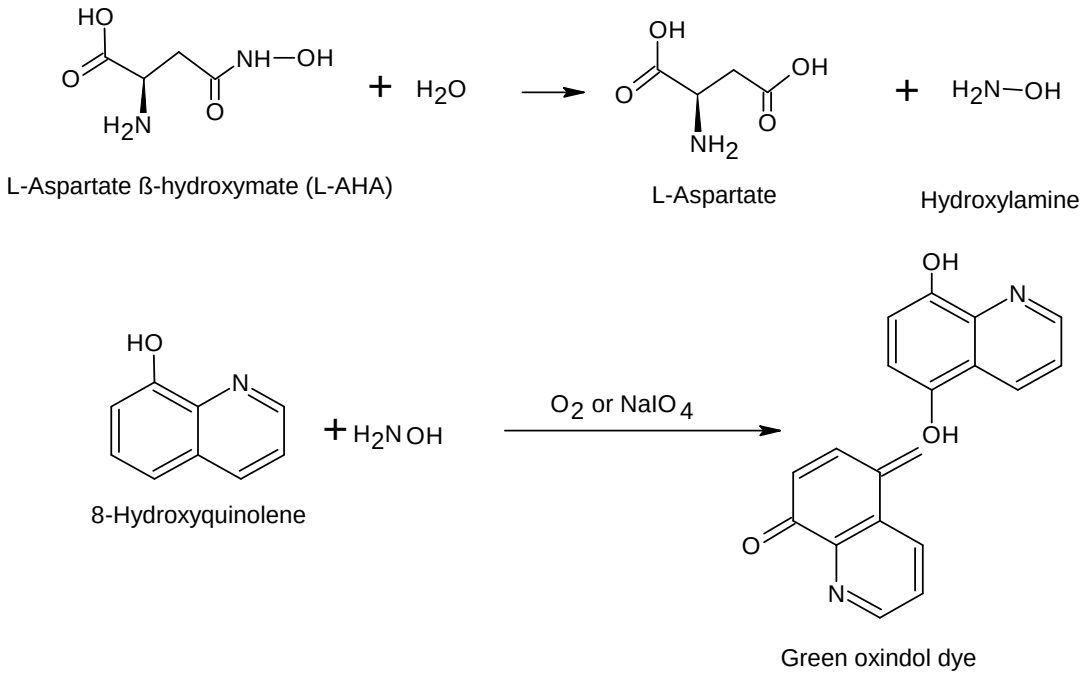
3.6 Enzyme assays

Bacterial cell pellet were suspended in respective enzyme assay buffer and sonicated (4 x 15 sec) for cell lysis in ice bath. The lysates were centrifuged at 14000 rpm for 10 min at 4ºC and clear supernatant were used for enzymatic estimations.

3.6.1 Glutaminase/asparaginase assay

Asparaginase activity was measured by L-aspartic acid β-hydroxamate (L-AHA) based assay. This assay relies on the reaction of hydroxylamine liberated from L-AHA with 8-hydroxyquinoline at high pH. The resulting green oxindol dye has an absorption coefficient of about 1.75 x 10 M^{-1} cm^{-1} at 705 nm, which can be detected with high sensitivity.

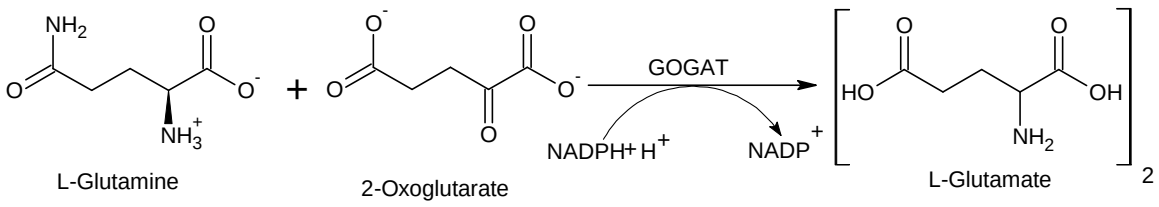
One unit of asparaginase activity is the amount of enzyme that catalyses the hydrolysis of 1 µmol substrate in 1 min at 25ºC. 20 µl enzyme solution was added to 30 µl of buffered 1 mM L-AHA and incubated for 60 min at room temperature. The reaction was stopped by addition of 240 µl of stop solution. The absorption was measured at 655 nm in a microplate-reader.



Reagents	Concentrations
Substrate colution	1 mM AHA in 50 mM MOPS (pH=7.0)
Na_2CO_3	1 M in ddH_2O
Chromogen	1% (w/v) hydroxyquinoline in DMSO
Oxidant	1% (w/v) $NaIO_4$ in ddH_2O
Stop solution	8 ml Na2CO3+1 ml chromogen+200 µl oxidant

3.6.2 Glutamate synthase (GOGAT) assay

Glutamate-synthase activity was measured according to the protocol defined by Meister (Meister 1985 and Meers *et al.* 1970). The activity by this method is measured on the basis of decrease in the NADPH absorption. NADPH has an absorption coefficient at 6220 L-mol^{-1}cm^{-1} at 340 nm.

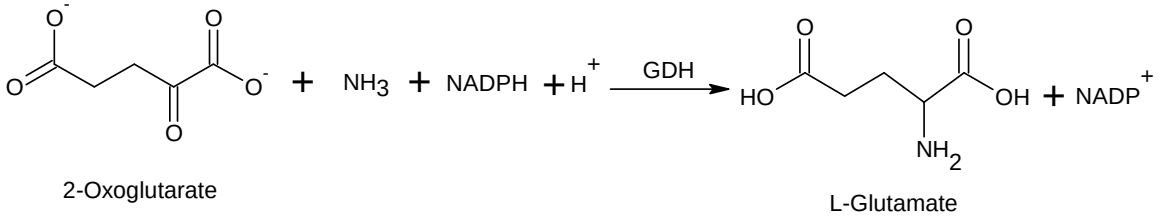


Reagents	Concentrations
L-Glutamine	10 mM dissolved in 100 mM Tris-HCl, pH=7.8
NADPH	0.35 mM in ddH_2O (Freshly prepared)
α-Ketoglutarate	5 mM in ddH2O, neutralize with NaOH (0.1N)

260 µl L-Gln, 10 µl NADPH and 10 µl α-Ketoglutarate was added to each well in the microtiter plate and finally 20 µl enzyme solutions were added to the wells to start the reaction. Blank was prepared with ddH_2O instead of enzyme solution. Decrease in absorbance was recorded at every 3rd min at 340 nm in micro-plate reader. One unit GOGAT oxidizes 1µM NADPH in 1 min under above defined conditions.

3.6.3 Glutamate dehydrogenase assay

Activity of glutamate dehydrogenase was measured by the protocol of (Sonawane *et al.* 2003a). This is a continuous spectrophotometric assay, based on the consumption of NADPH by the enzyme which converts α-ketoglutarate to L-glutamate in the presence of co-enzyme NADPH and ammonium ions.



2-Oxoglutarate

L-Glutamate

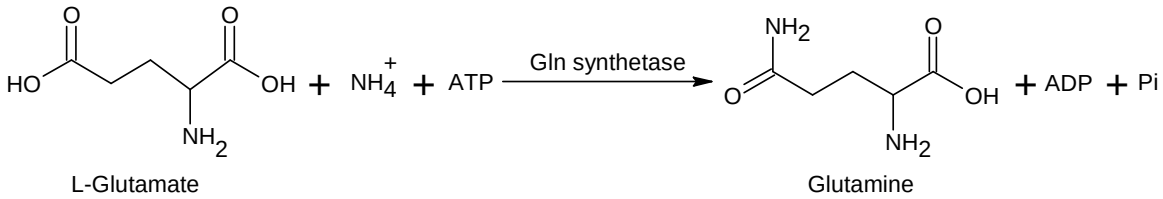
Reagents	Concentrations
Assay buffer	0.1 mM Imidazole, pH=7.3
α-ketoglutarate	0.2 M in ddH2O, neutralize with NaOH (0.1N)
Ammonium acetate	13 M in ddH_2O
NADPH	10 mg/ml in ddH_2O
ADP	0.1 M in ddH_2O

249 µl assay buffer was added in each well of micro-plate followed by addition of 20 µl α-ketoglutarate, 5 µl Ammonium acetate, 3 µl NADPH and 3 µl ADP. The reactions were started by

addition of 20 µl enzyme solutions, the decrease in absorption was measured at 340 nm for 5 min. Blank was prepared in the same way but instead of enzyme solution water was used. One unit of GDH oxidizes 1 µM of NADPH per min under described conditions.

3.6.4 Glutamine synthetase assay

Activity of enzyme GS was measured by the protocol of (Gawronski & Benson 2004). This end point assay is based on the detection of inorganic phosphate released from hydrolysis of ATP by GS in presence of L-glutamate and ammonium ions.



L-Glutamate Glutamine

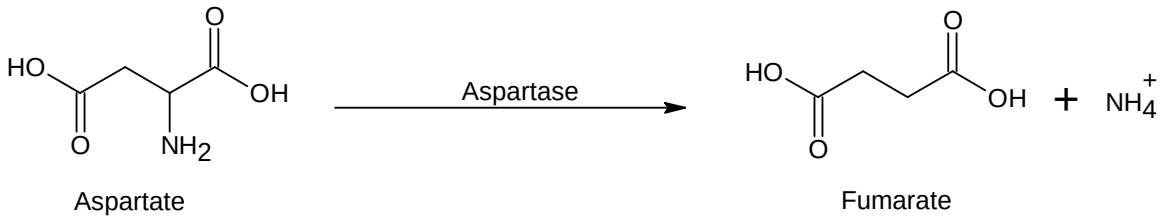
Reagents	Concentrations
Assay buffer	0.1 M MOPS, pH=7.5, 50 mM $MgCl_2$, 250 mM L-Glutamate, 50 mM NH_4Cl and 10mM ATP (freshly made) final pH adjusted to 7.5.
Phosphate standard	20 mM potassium phosphate dibasic in ddH_2O
Solution A*	12% (w/v) ascorbic acid in 1 N HCl
Solution B*	2% (w/v) ammonium molybdate tetrahydrate in ddH_2O
Solution C#	Mixture 2:1 (solution A + solution B)
Solution D	2% (w/v) sodium citrate tribasic dehydrate + 2% (v/v) acetic acid in ddH_2O

*Solutions were stable at 4ºC for 1 week, #solutions were mixed freshly just before use and stored in ice.

20 µl enzyme solutions were pipetted in micro-plate and the reaction was started by addition of 50 µl assay buffer at room temperature for 10 min. The reaction was terminated by addition of 150 µl of solution C. The low pH of solution C, terminates the reaction. Exactly 5 min later, 150 µl solution D was added to stop the color development. The reaction mix was allowed to equilibrate at RT for 15 min. The absorbance was measured at 655 nm in a plate reader. Standard phosphate (0-20 mM) was also prepared and treated same as samples. The blank was made by addition of 20 µl ddH_2O instead of enzyme solution.

3.6.5 Aspartase assay

Aspartase was measured by the protocol defined by (Williams & Lartigue 1969). This enzyme can hydrolyze L-aspartate into fumarate. The increase in absorbance was measured at 240 nm as fumarate has extension coefficient 2530 L-mol^{-1}cm^{-1} at 240 nm.



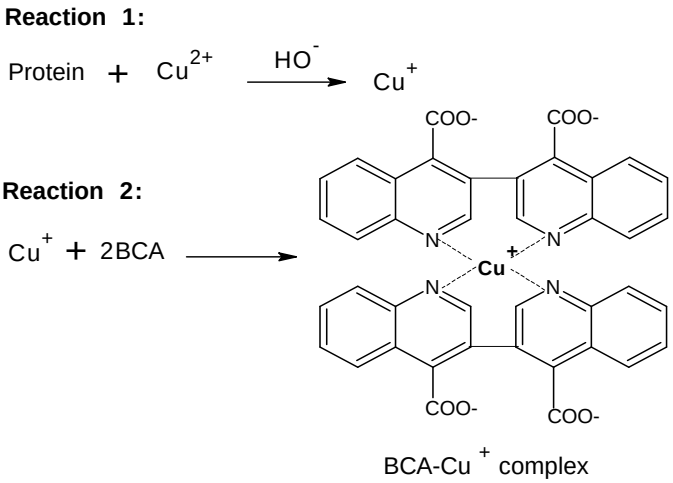
Aspartate Fumarate

Reagents	Concentrations
Tris-HCl	150 mM ris-HCl, pH=8.5
$MgCl_2$	60 mM in ddH_2O
EDTA	3 mM in ddH_2O (add 0.1N NaOH to dissolve)
Aspartic acid	500 mM
Assay buffer	Mix 50 ml Tris-HCl buffer with 5 ml $MgSO_4$, 5 ml EDTA and 15 ml L-aspartate, ddH_2O was added to make up to 100 ml.

The assay buffer was equilibrated at 30ºC. 100 µl enzyme solutions were added to the 900 µl assay buffer in 1 ml cuvette. The change in OD was recorded at 240 nm on spectrophotometer (Ultrospec 3000, Amersham Biosciences). Blank was also prepared with water. One unit aspartase converts 1 µM of L-aspartate to fumarate per minute at 30ºC.

3.7 Protein estimation (BCA method)

Total protein in bacterial pellets/purified protein was estimated by using Bicinchoninic acid assay kit (Pierce, USA). The standard BSA was also prepared in the same buffer in which, bacterial pellets lysed or protein solution present. This method combines well known reduction of Cu^{++} to Cu^{+} by protein in an alkaline medium and make tetradentate-Cu^{+} complex. One Cu^{+} is chelated by 2 molecules of BCA which produces purple colored complex (BCA-Cu^{+}) have strong absorbance at 562 nm. This method is sensitive till 1µg of protein.



Reagents	Concentrations
Reagent A	Sodium carbonate, sodium bicarbonate and bicinconinic acid and sodium tartrate in 0.1 M NaOH
Reagent B	4% (w/v) cupric sulfate in ddH_2O
Working solution	50 part of reagent A + 1 part of reagent B
BSA standard	1 mg/ml (freshly prepared)

The standard BSA was pipetted in micro-plate in triplicate along with samples, the volume of each well was kept equally by addition of buffer or ddH_2O. 200 µl freshly prepared working solution was added to the micro-plate by multi-channel pipetter. The plate was incubated at 37ºC for 30 min, cooled at RT for 5 min and absorbance was measured at 560 nm. Blank was prepared with ddH_2O.

3.8 Amino acid estimation by HPLC

Amino acid (Glu, Gln, Asp and Asn) were measured by using O-pthalaldehyde (OPA) pre-column derivatization method as described by (Suresh *et al.* 2002). The method is based on alkylation of –SH groups prior to OPA derivatization of amino acids and separation by reverse phase chromatography. The derivatized amino acids ultimately can be detected by fluorescence detector with excitation at 330 nm and emission at 450 nm. The HPLC system (Merck-Hitachi, Darmstadt) consisted of L-7100 pump, AS-2000A auto-sampler, Schimazdu RF-551 Fluorescence detector, D-7500 integrator and L-7612 solvent degasser.

Reagents	Concentrations
Sodium borate buffer	0.5 M, pH=10.5
Ethanol	80% (v/v) HPLC grade
OPA reagent (Freshly prepared)	10 mg/ml, in methanol: borate buffer (1:9) + 0.01% ß-mercaptoethanol
Buffer A	25 mM sodium acetate, pH 6.8
Buffer B	methanol

3.8.1 Pre-column derivatization by OPA

The samples collected as described in section (3.4.1). 100 µL supernatant were used to assay residual amino acid levels, whereas the cell pellet were used to measure intracellular amino acid concentrations. The pellet were suspended in 200 µl 80% ethanol and incubated at 100°C for 10 min. After centrifugation at 14,000 rpm, 100 µl samples were derivatized by addition of 50 µl sodium borate buffer and freshly prepared O-pthalaldehyde reagent (25 µl). The samples were incubated at 25°C for 10 min and finally the volume was adjusted to 1 ml with water (these samples can be stored for 1 week at -20°C), standard amino acids were treated in the same way.

3.8.2 HPLC analysis

Dilution of each derivatized sample (1:50) were made with mobile phase (95% A + 5% B), centrifuged briefly and 40 µl was injected to equilibrated LichroSphere® 60RP Select B, reverse phase analytical column (5 µm particle size, 4 mm diameter and 250 mm long).

Time (min)	Solvent A (%)	Solvent B (%)
0.0	95	5
5.0	95	5
20.0	75	25
25.0	0	100
27.0	0	100
29.0	95	5
32.0	95	5

The temperature of column was maintained at 40°C. Amino acids were eluted at constant flow rate of 1.0 ml/min with the gradient as described below in the table.

3.9 Isolation of bacterial DNA

3.9.1 Isolation of genomic DNA Sigma (Genelute™ bacterial genomic DNA kit)

Genomic DNA from *P. putida* cells was isolated by using Genelute™ bacterial genomic DNA kit. The cells were grown overnight in 5 ml appropriate medium at 30°C, 1.5 ml culture was transferred to microfuge tube and cells were collected at 10000 rpm for 1 min. Pellet were suspended in 180 µl lysis solution T, 20 µl RNAse A was added, vortexed and stored at RT for 2 min. 20 µl proteinase K was added, mixed and incubated at 55°C for 30 min. Subsequently, 200 µl lysis solution C was added, vortexed and incubated for another 10 min at 55°C. Absolute ethanol (200 µl) was added to the bacterial lysate, votexed briefly and loaded to the binding column (prepared according to kit instructions). Column was centrifuged at 10000 rpm for 1 min, washed with 500 µl Wash Solution 1 and followed by washing with 500 µl Wash Solution Concentrate. Column was dried by centrifugation at 12000 rpm for 3 min. Finally 200 µl sterile ddH_2O was added to the column and centrifuged at 10000 rpm for 1 min to elute DNA.

3.9.2 Isolation of plasmid DNA Sigma (Genelute™ miniprep kit)

Plasmid DNA from *E. coli* cells was isolated by using genelute miniprep kit. The cells were grown overnight in 5 ml LB medium with antibiotics at 37°C, 2 ml culture was transferred to microfuge tube and cells were collected at 10000 rpm for 1 min. Pellet were suspended in 200 µl Resuspension Solution containing RNAse A, vortexed to mix and 200 µl Lysis Solution was added. The mixture was mixed by inverting and the tube was allowed to stand for 5 min to clear the solution. 350 µl Neutralize Solution (S3) was added, inverted 4-6 times to mix and centrifuged at 14000 rpm for 10 min. Clear supernatant was loaded in column (prepared according to manual instruction), spinned at 12000 rpm for 1 min and flow through was discarded. The column was washed with 750 µl wash solution and spinned 2 min to dry. The plasmid DNA was eluted with 100 µl ddH_2O. The purity and quantification were performed by using absorbance at A_{260}/A_{280} in spectrophotometer.

3.9.3 Phenol-chloroform extraction

Equal volume of Roti-Phenol/chloroform loesung (Roth) was added in DNA solution and mixed by vortexing. The mixture was centrifuged for 5 min at 14000 rpm. The clear aqueous phase was transferred to new tube and 1/20 volume of 5 M NaCl was added, followed by addition of two volumes of absolute ethanol. Mixed by vortexing and incubated at 4°C for 10 min. Precipitated DNA was collected at 14000 rpm for 10 min at 4°C, washed with 500 µl of 70% (v/v) ethanol and again centrifuged. Pellet were dried in SpeedVac and dissolved in desired volume of TE or ddH_2O.

3.9.4 DNA quantification and purity estimation

The purity and quantification of DNA were performed by using absorbance estimation at A_{260} and A_{280} in spectrophotometer. 2 µl of genomic/plasmid DNA was added to 50-100 µl ddH_2O and loaded on quartz cuvette. The absorbance at 260 nm was used for calculation of DNA concentration and the ratio A_{260}/A_{280} was calculated to judge the purity of DNA samples.

DNA concentration (µg/ml) = (A_{260}) x (dilution factor) x (50 µg DNA/ml)/(1 OD_{260} unit)

The ratio of A_{260}/A_{280} between 1.65 to 1.85 corresponds for pure DNA.

3.10 DNA analysis

3.10.1 Agarose gel electrophoresis (Maniatis *et al.*, 1982)

DNA molecules can be separated on the basis of size and charge by using agarose gel electrophoresis. DNA molecules are negatively charged and move from cathode to anode in electric field which is the basis of electrophoresis. Particular pore size matrix of agarose gel is used to separate the DNA molecules according to their size, as the electrophoretic mobility of DNA fragments is inversely related to their length. Agarose gels have a large range of separation, but relatively low resolving power.

Reagents	Concentrations
TAE (50X), per liter	242 g Tris-HCl, acetic acid 57.1 ml, 0.5 M EDTA (100 ml), pH=8.0
Agarose solution	0.5 to 2% (w/v) in 1X TAE
Ethidium bromide	10 mg/ml (stock)
Gel loading dye solution (6X)	0.25% bromophenol blue + 0.25% (w/v) xylene cyanol FF in ddH_2O + 30% (v/v)glycerol in ddH_2O
Electrode buffer	1X TAE

By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using this technique. Bands of DNA in gel can be easily detected by staining with ethidium bromide which inter-chelate with DNA and fluoresces under UV light.

Agarose powder according to requirement was weighed and mixed with 50 ml of 1X TAE for mini gel and 100 ml 1X TAE for large size gel. Boiled for a while to dissolve agarose in a microwave oven, cooled for 10 min and 5-10 µl ethidium bromide solution (10 mg/ml) was added. Gel solution was poured in casting cassette fixed with (0.5-1 mm) comb, allowed for 20 min to solidify. Electrode buffer was filled in unit according to requirement and 10 µl DNA solution (>50 ng) with 2 µl loading dye were loaded on wells along with standard known markers. The gel was subjected to run at 80V-120V until the front dye reached before 1 cm of the end and visualized in UV transilluminator at 365 nm. Photographs were taken by using Kodak DC-120, CCD camera and documentation system.

3.10.2 DNA extraction from agarose gels, Qiagen (QIAquick Gel extraction kit)

DNA bands stained with ethedium bromide were excised from agarose gel and gel slices were weighed in 2 ml microfuge tube. Buffer QG (3 times to the gel weight) was added to the tube and incubated at 50^{o}C for 10 min, in between 2-3 times mixed by vortexing. To increase the yield, one volume iso-propanol was added and mixed well. The sample was loaded on QIAquick column and centrifuged for 1 min at 10000 rpm, flow through was discarded. Column was washed with 0.75 ml Buffer PE and dried for 1 min at 10000 rpm. DNA was eluted by using 50 µl ddH_2O to the column and centrifugation at 10000 rpm for 1 min.

3.10.3 Sequencing (urea:polyacrylamide) gel electrophoresis of DNA

Urea containing polyacrylamide gels are commonly used for manual sequencing of DNA. 8-10% urea acrylamide gels were used for sequencing of 50-200 bp long DNA in our experiments. Bio-Rad DNA sequencing gel unit was used for DNA sequencing.

21 x 40 cm Glass plates were washed thoroughly using detergent under tap water, ringed by distilled water for at least 3 times and fitted in cassette with 0.4 mm thick spacers. Small glass plate was siliconised by using (Sigmacoat) solution just before assembling. 100 ml gel solution was

prepared and poured according to instructions given in manual supplied with Sequi-Gen® GT System of Bio-Rad sequencing unit.

Reagents	Concentrations
10X Tris-Borate-EDTA (TBE) buffer (1 lt)	108 g Tris base, 55 g boric acid, 9.3 g EDTA, pH 8.0
Polyacrylamide solution	19 % acrylamide, 1 % bisacrylamide
8% Urea : acrylamide (1 lt)	80 ml polyacrylamide solution, 480 g urea (ultrapure), 100 ml of 10X TBE, water up to 1 lt (stored at 4^{o}C)
APS	10% (w/v)
DNA Loading buffer	91,6 % formamide, 17 mM EDTA pH 8,0 0.05 % bromophenolblue and 0.05% xylenecyanol

Composition of sequencing gel

Reagents	Amount
8% Urea : Polyacrylamide	100 ml
10% APS	750 µl
TEMED	75 µl

After polymerization the gel plates were fitted in the unit and 0.5X TBE was filled over in upper and lower chambers. The gel was tempered at 55°C by pre-run at 2200 volt, 60 W for 30 min. The samples were loaded and continue to run at 1800 volts, 55 W until the dye front crossed 2/3 part of the gel length. The gel was cooled at RT for 30 min, transferred to Whatman paper sheet, covered with Saran wrap and dried under vacuum at 85°C for 2 h. The dried gel was exposed to phosphor screen for 7 days and analyzed by phosphor imager.

3.10.4 Preparation of sequencing ladders

Chemical cleavage by base modification approach based on Maxam-Gilbert sequencing method, was modified by Bencini *et al* in (1984). This is a rapid method for chemical DNA sequencing which is commonly used as ladder for foot-printing reactions or for sequencing of short DNA oligonucleotides. Around 200000 cpm, ^{33}P labeled DNA probe was taken into 2 tubes along with 1 µg salmon sperm DNA, one tube was labeled as G+A ladder and another A+C ladder. In G+A tube

1 µl formic acid was added and incubated at 37°C for 30 min followed by addition of 150 µl piperidine and stored on ice. In another A+C tube 1 µl of 30% NaOH was added and incubated at 90°C for 15 min. 150 µl piperidine was added and stored on ice.

Reagents	Concentrations
Na/H Formate	1 M formic acid, pH 2 (NaOH used for pH adjustment)
Piperidine	1:10 (v/v) diluted solution in ddH_2O
NaOH	30% (w/v) in ddH_2O
Ethanol	70% (v/v) in ddH_2O
Salmon sperm DNA	1 µg/µl in ddH_2O
SDS	1% (w/v) in ddH_2O

The cleavage reaction was carried out by incubating both tubes at 90°C for 30 min. 150 µl of 70% ethanol was added with Tube A+C. The tubes were filled with 1 ml butanol, vortexed and centrifuged to collect pellet. Pellet were washed by addition of 150 µl of 1% SDS and 1 ml butanol, centrifuged for 5 min and dried in speedvac. Dried pellets were suspended in 5 µl DNA loading buffer. Ladders were stored at -20°C if not loaded immediately.

3.11 Protein Analysis

3.11.1 Native polyacrylamide gel electrophoresis (Native-PAGE)

Native PAGE is a widely used method for protein analysis in natural state. Proteins are folded into its 3D structure which can be only maintained under natural conditions. This method is frequently applied in the analysis of DNA-protein interaction, protein-protein interaction and protein-ligand interaction studies.

Reagents	Concentrations
4X Tris-glycine buffer	100 mM Tris-HCl (pH 8,6) and 800 mM Glycine
Acrylamide solution	30 % acrylamide, 0.8 % bisacrylamide
APS	10% (w/v)
Running buffer	25 mM Tris-HCl (pH 8,6) and 200 mM Glycine
5X Sample buffer	50 mM Tris-HCl (pH 6.8), 0.01% (w/v) bromophenol blue, 25% (v/v) glycerol

Composition of Native gel (6%)

Reagents	Amount (10 ml)
4X Tris-glycine buffer	2.5 ml
Acrylamide solution	2.0 ml
Water	5.4 ml
APS	0.1 ml
TEMED	0.008 ml

Glass plates were cleaned by detergent, washed thoroughly under tap water and ringed with milli-Q water. Plates were dried and wiped with ethanol. Gel solution was poured in between 2 glass plates separated by 1 mm spacers and comb was inserted before polymerization. 20 µl samples were mixed with 5 µl of sample buffer and loaded in the wells by micro-syringe. Gel was run at 20 Volt/cm for 30 min and analyzed accordingly by coomassie staining or by autoradiography if, radioactivity is used.

3.11.2 SDS-Polyacrylamide gel electrophoresis

Proteins can be separated on the basis of their mass by using denaturing polyacrylamide gel electrophoresis. Sodium dodecyl sulphate (SDS), an anionic detergent that binds with protein molecules and denature it by disrupting non-covalent interactions. One SDS molecule binds with every two amino acids therefore; the complex of SDS with the denatured protein carries a large net negative charge and migrates towards the anode. The velocity of migration of SDS-protein complexes is inversely proportional to the mass of the protein.

Reagents	Concentrations
Acrylamide solution	30 % acrylamide, 0.8 % bisacrylamide
Resolving buffer	1.5 M Tris-HCl, pH 8.8
Stacking buffer	0.5 M Tris-HCl, pH 6.8
APS	10% (w/v)
Loading buffer	0.1 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 3 % mercaptoethanol, 0.01% (w/v) bromophenol blue, 10% (v/v) glycerol
10 X running buffer	250 mM Tris, 1.9 M glycine, 1 % (w/v) SDS

Composition of SDS gel

Reagents	15% Resolving gel solution (10 ml)	4.5% Stacking gel solution (5 ml)
Acrylamide solution	4,000 µl	650 µl
Resolving buffer	2500 µl	-
Stacking buffer	-	1250 µl
SDS; 10 % (w/v)	100 µl	50 µl
Water	3350 µl	3050 µl
APS;10 % (w/v)	50 µl	25 µl
TEMED	25 µl	10 µl

Resolution gel was prepared and poured in between 2 glass plates separated by 1-2 mm spacers. After polymerization stacking gel was poured above resolution gel and comb was inserted before polymerization.

Protein solution was prepared by mixing 1:1 (v/v) ratio of loading buffer 95°C for 3 min. For protein analysis of bacterial cells; 1 ml culture pellet was suspended in 200 µl of 50 mM Tris-HCl buffer, pH 7.6. The resulting suspension was mixed with 50 µl loading buffer and incubated at 95°C for 3 min. Samples were centrifuged at 14000 rpm for 5 min before loading. The electrophoresis unit was filled with running buffer and comb was removed. Equal amount of samples were then loaded into the wells with a micro-syringe. Separation of the proteins was carried out at the 50 V for 30 min. Once proteins entered into the separation gel the current was set at 200 V and allowed to run for additional 45 min. Finally, the proteins in the gel were visualized by staining with Coomassie blue.

3.11.3 Coomassie staining

Reagents	Concentrations
Staining solution	45% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Brilliant Blue
Destaining solution	45% (v/v) methanol, 10% (v/v) acetic acid

After electrophoresis, the separating gel was immersed in the staining solution for 20 min by gentle shaking at RT. Gel was de-stained after rinsing with water for 1-2 h with destaining solution. During de-staining the destaining solution was changed 2 times. The de-stained gel was dried in vacuum dryer.

3.12 Polymerase chain reaction

Polymerase chain reaction (PCR) is an effective tool for amplifying specific regions of DNA strand which may be a single gene, just a part of gene, or a non-coding sequence. The reaction involves denaturation of template DNA, annealing of primers to its complementary regions and extension for synthesis of new DNA strand. This reaction completely relies on temperature control by using an automated thermal cycler. Thermo stable DNA polymerase (*Taq* polymerase) from *Thermus aquaticus* is used to increase efficiency of PCR. This enzyme has high 5`-3` processivity but lack of 3`-5` exonuclease activity. There are many other choices to use DNA polymerases which have efficient stability at high temperature and 3`-5` exonuclease activity (proofreading activity). *PfuTurbo* DNA polymerase (Stratagene) can amplify complex genomic targets up to 10 kb and vector targets up to 19 kb in length with high fidelity. PCR is now days used in many DNA protocols like gene amplification, mutagenesis, finger printing, automated sequencing and several diagnostic purposes.

The reaction setup was made in 50 µl total reaction volume containing 1X *Taq* buffer, dNTP mix for new strand synthesis, forward and reverse primers, template DNA, DNA polymerase enzyme and the volume was maintained up to 50 µl by using PCR grade water.

Reagents	**Amount**
10X DNA polymerase buffer	5 µl
2 mM dNTPs mix	5 µl
Forward primer	1 µl (50 pico mole)
Reverse primer	1 µl (50 pico mole)
Template DNA	50-200 ng
Taq polymerase/*PfuTurbo* polymerase	1 µl (2.5U)
PCR water	Upto 50 µl

The denaturation temperature was 95°C for all DNA samples but the timing was different as genomic DNA the very first denaturation time was at least 4-5 min but for plasmid DNA it was only 30 sec. The annealing temperature and GC content of primers were matched and calculated before designing. The annealing temperature in cycles was set at 5°C less than primer Tm and annealing time was normally 30-40 sec. Polymerase activity of Taq or *PfuTurbo* is maximum at 72°C, so it was extension temperature in all reactions. The time of extension was adjusted according to length of amplifying DNA as described in DNA polymerase manual. Sometimes $MgCl_2$ concentration was optimized when amplification was poor.

Cycles	Denaturation	Annealing	Extension
Denaturatuion	3-5 min at 95°C	---	---
Cycles 25-30	1 min at 95°C	30-40 sec at 45-65°C	30-60 sec at 72°C
Last cycle	---	---	5-8 min at 72°C

Negative control reaction without template DNA was also made. 5-10 µl amplified sample was analyzed by agarose gel electrophoresis. DNA marker was loaded along with samples to verify the size of amplified fragment. Gels were stained in ethidium bromide to visualize DNA bands.

3.13 DNA digestion with restriction enzymes

Restriction endonucleases are enzymes that cleaves sugar-phosphate backbone of double stranded DNA at specific sequences leaving phosphate group on 5` end and hydroxyl group on 3` end. These enzymes can either cut DNA at the same point in both strands to produce blunt ends or cut asymmetrically which produces cohesive ends. One unit of restriction enzyme can digest 1 µg of λ-DNA in 1 h at 37°C. For restriction digestion of vector or cloned DNA fragments, reaction mixture (20 - 50 µl volume) containing 0.1 to 1 µg DNA, 20 to 30 U restriction enzyme and 1/10 volume of 10X reaction buffer were incubated at 37°C for 3-5 h. The restriction enzymes were purchased from Roche Diagnostics, Germany. Double digestion by restriction enzymes was performed according to buffer range selection as defined in Roche catalogue for optimum activity of restriction enzymes. Digested DNA was purified by gel extraction or direct purification kits.

3.14 Dephosphorylation of 5`-phosphate group

Alkaline phosphatase catalyzes removal of 5`-phosphate groups from DNA and RNA. Calf intestinal phosphatase (CIP) treated vectors are needed by T4-DNA-ligase enzyme to prevent the self ligation. One unit of CIP is defined as the amount of enzyme that hydrolyzes 1 μM of p-nitrophenylphosphate or p-nitrophenol in 1 min at 37°C. dephosphorylation reaction was carried out in 1X reaction buffer containing 0.1 μg to 1 μg DNA fragment or digested vector. 2-3 units of CIP were added and incubated at 37°C for 30 min.

3.15 DNA ligation

T4-DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5`-phosphate and 3`-hydroxyl termini in duplex DNA or RNA. Ligation reaction was performed in 1X T4-DNA ligase buffer containing 50 ng vector DNA and 200 ng DNA fragment. 2U of T4-DNA ligase was added and the reaction mixture was incubated at 22°C for 1 h. Ligase was heat inactivated at 65°C for 10 min, before transformation of recombinant vector to *E. coli* cells.

3.16 Gene replacement

Gene targeting by homologous recombination is a genetic tool that permits modification of cellular genes in a precise and predominant fashion. Targeting knockout of genes involves first designing and construction of an appropriate targeting vector in which the gene of interest have been disrupted with a positive selectable marker. The second step involves introduction of targeting vector into the bacterial culture followed by selection of those cells in which internal selectable marker has become integrated into the genome of bacteria.

pK18 vector was used for targeted gene knockout. This is a small (2.6 kb) multi-copy vector (Pridmore, 1987). The vector contain pUC lacZα-complementation peptide, Kannamycin resistance gene, pUC18 and pUC19 cloning sites. The plasmid and their derivatives allow simple and rapid transfer of inserts from one replicon to another.

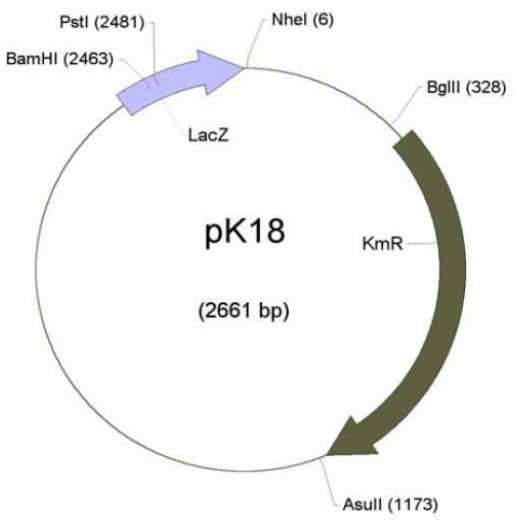


Figure 3.1 Map of pK18 plasmid, *lacZ* and Km^R genes are represented by arrow showing the direction of transcription.

3.16.1 Replacement of gene *aatP*

In order to knock out ABC transporter genes PP1068 (*aatP*), a 261 bp internal fragment of the gene (147-408 bp relative to translation start site) from *P. putida* KT2440 genomic DNA was amplified using primers PP1068_For and PP1068_Rev. The amplified fragment was cloned in pK18 vector to generate pK18*aatP* vector, which was electroporated into *P. putida* KT2440 followed by kannamycin resistant clone selection named as KT*aatP* mutants. Integration of denatured *aatP* in mutants was confirmed by using primer pK18_For (designed for vector region) and another primer PP1068_361 (designed for gene *aatP*), which produced 289 bp fragment.

3.16.2 Replacement of gene *aatJ*

Gene PP1071 (*aatJ*) was inactivated by using same strategy a 284 bp internal fragment (189 bp to 473 bp) was amplified by using the primers PP1071_For and PP1071_Rev. The amplified fragment was ligated with pK18 vector to produce pK18*aatJ*. This vector was electroporated into *P. putida* KT2440 cells ultimately the mutants produced were named as KT*aatJ*. The vector pK18 is a suicidal vector hence can not multiply inside *P. putida* mutants and integrate to the chromosome of *P. putida* KT2440 by means of homologues recombination. Integration of denatured gene fragment was verified by using primers, pK18_For and PP1071_414, which gave 304 bp amplified fragment.

3.17 Expression and purification of recombinant proteins

3.17.1 Expression and purification of AauR

The gene encoding AauR (PP1066) in *P. putida* KT2440 was completely amplified by PCR using specific primers aauRFor and aauRRev by using PCR program (95°C initial denaturation for 4 min, 95°C denaturation for 1 min, 62°C annealing for 40 sec, 72°C extension for 1 min and final extension at 72°C extension for 8 min until 25 cycles.). The amplified gene was ligated with pTrcHis-A expression vector (Invitrogen) to generate the vector pTrc*aauR*. The sequenced recombinant vector was finally transformed into *E. coli* BL21. 6XHis-AauR was over expressed from *E. coli* BL21 containing pTrc*aauR*.

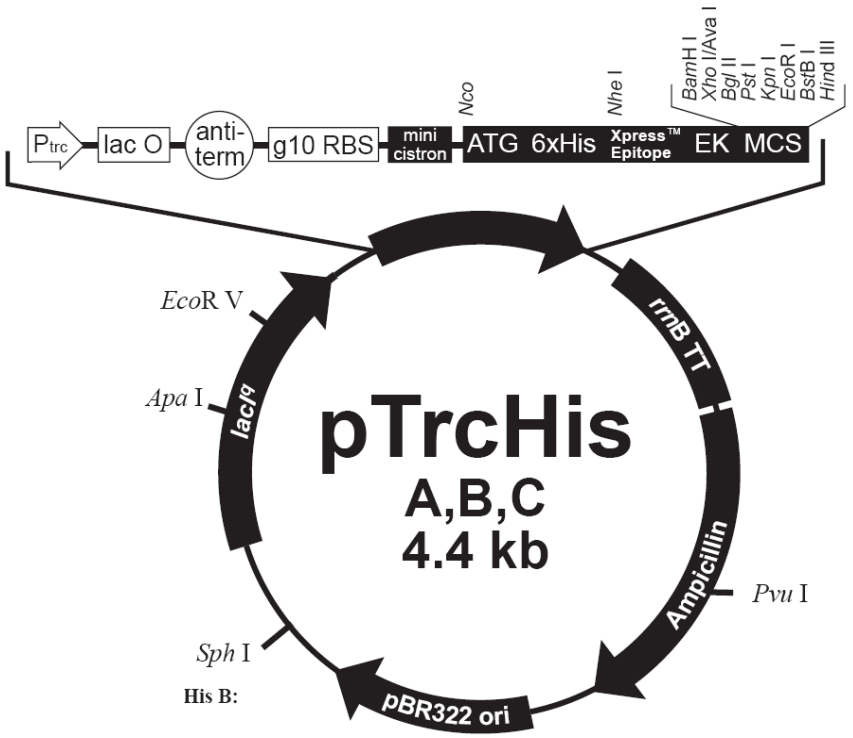


Figure 3.2 Plasmid vector pTrcHis, Amp^R, *ori* pBR322, *trc* promoter, $lacI^q$, 6x His-tag at N-terminal position (Invitrogen).

Single colony was grown overnight with 20 ml LB Amp at 37°C, added to 1 liter LB Amp medium and grown at 37°C with 200 rpm agitation until OD A_{600} reaches 0.8 to 0.9. The expression was induced by addition of 1 mM IPTG and grown additionally for 6 hrs at 200 rpm in 37°C. Cells were harvested at 5000 rpm for 10 min at 4°C, washed with 100 ml cold His-tag protein binding buffer (20 mM HEPES, pH 7.5 with 500 mM NaCl and 100 mM imidazole) and resuspended with 20 ml

of the same buffer. The cell pellets were disrupted by sonication for 10 min with 15 s pulse, 15 s pause at 80 cycles (Bandelin Sonoplus) cooled on ice, followed by centrifugation at 20,000 rpm for 30 min. The supernatant was loaded on equilibrated Ni-NTA column (HisTrap^{TM}-FF, GE Healthcare Biosciences) and protein was eluted with linear gradient of imidazole (100-500 mM). AauR fractions were pooled together and purified by gel filtration using Superdex 200^{TM} (Amersham Biosciences) analytical column, under same buffer conditions. The purity was also judged by 12% SDS-PAGE. Finally protein AauR was dialysed against buffer (50 mM HEPES, pH 7.5 and 500 mM NaCl), concentrated by centricon concentrator (10kD MWCO, Millipore Inc), mixed with 10% (v/v) glycerol and stored at 4°C.

3.17.2 Expression and purification of AauS

The gene PP1067 of *P. putida* KT2440 was amplified from 1023-1905 bases (relative to translation start site) by omitting trans-membrane domain, by PCR using specific primers aauSFor and aauSRev by using PCR program (95°C initial denaturation for 4 min, 95°C denaturation for 1 min, 60°C annealing for 40 sec, 72°C extension for 1 min and final extension at 72°C extension for 8 min until 25 cycles). The amplified gene was ligated with pTrcHis-A expression vector (Invitrogen) to generate the vector pTrc*aauS*. The sequenced recombinant vector was finally transformed into *E. coli* BL21. 6XHis-AauS was over expressed from *E. coli* BL21 containing pTrc*aauS*. Single colony was grown overnight with 20 ml LB Amp at 37°C, added to 1 liter LB Amp medium and grown at 37°C with 200 rpm agitation until OD A_{600} reaches 0.8 to 0.9. The expression was induced by addition of 1 mM IPTG and grown additionally for 6 h at 200 rpm at 37°C. Cells were harvested at 5000 rpm for 10 min at 4°C, washed with 100 ml cold His-tag protein binding buffer (50 mM Tris-HCl, pH 7.5 with 500 mM NaCl and 70 mM imidazole) and resuspended with 20 ml of the same buffer.

The cell pellets were disrupted by sonication, followed by centrifugation at 20,000 rpm for 30 min. The supernatant was loaded on equilibrated Ni-NTA column and bound AauS was eluted with linear gradient of imidazole (100-500 mM). AauS purity was also judged by 12% SDS-PAGE. Finally protein AauS was dialysed against buffer (50 mM Tris-HCl, pH 7.5 containing 500 mM NaCl) and concentrated by centricon concentrator, 10% (v/v) glycerol was added and stored at -20°C.

3.18 Site directed mutagenesis

In vitro site-directed mutagenesis is an invaluable technique used for carrying out vector manipulation for studying protein structure-function relationships. QuickChange site directed mutagenesis protocol can be applied in any plasmid vector for deletion, insertion of single or multiple amino acids. This is a simple and four step procedure, which generate mutants with high efficiency.

The QuikChange site-directed mutagenesis method is performed using high fidelity *PfuTurbo*® DNA polymerase. *PfuTurbo* DNA polymerase replicates both plasmid strands. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuTurbo* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid. Following temperature cycling, the product is treated with *Dpn* I endonuclease which is specific for methylated and hemi-methylated DNA and is used to digest the parental DNA template at the (target sequence: 5´-Gm6ATC-3´). The nicked parental vector is digested by bacterial endonucleases leaving intact mutated vector. Vector DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion.

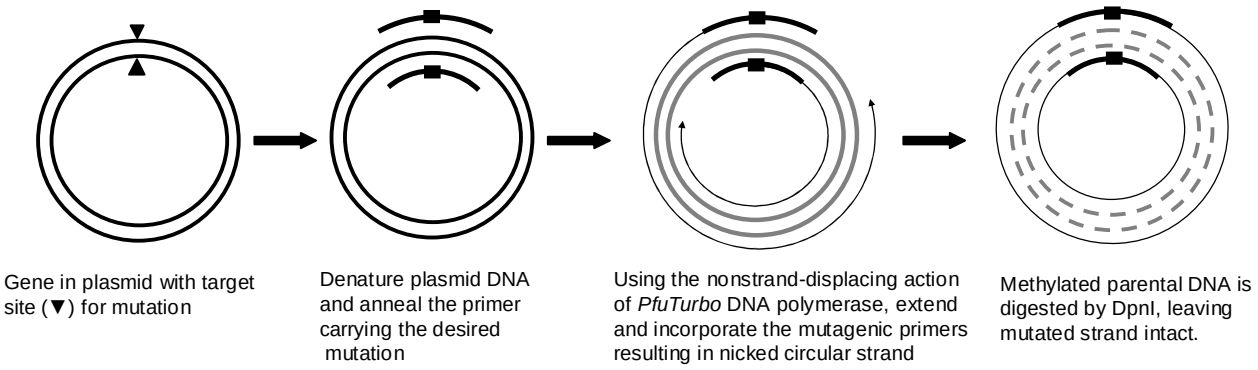


Figure 3.3 Overview of the QuikChange® site-directed mutagenesis mechanism.

3.18.1 Expression vector construction and Site directed mutagenesis in *aatJ*

Mutation of pFLAG*aatJ* template was performed in 50 µl PCR reaction mix containing 50 ng template DNA, 150 ng forward primer and 150 ng reverse primer, 200 µM dNTP mix and 2.5U

PfuTurbo DNA polymerase in 1x *PfuTurbo* buffer. The PCR reaction was performed as; 95°C initial denaturation for 30 sec, 95°C denaturation for 30 sec, 58°C annealing for 1 min and 68°C extension for 8 min until 16 cycles. 5 µl *Dpn* I buffer and 10U of *Dpn* I was added to PCR mixture and incubated at 37°C for 1h. The control reaction was prepared in same way by omitting only *PfuTurbo* DNA polymerase. The resultant PCR mixture (10 µl) was used to transform *E. coli* Top10. Sequenced and verified vectors were finally used for expression in *E. coli* BL21 cell.

3.18.2 Over-expression and purification of AatJ and mutated variants of AatJ

The gene PP1071 (72-918 bp excluding signal sequence) from *P. putida* KT2440 genome was amplified by PCR using primers PP1071_For and PP1071_Rev. The amplified fragment was ligated with pFLAG-ATS vector to produce pFLAG*aatJ* recombinant vector. The sequenced pFLAG*aatJ* vector was used as template for site directed mutagenesis.

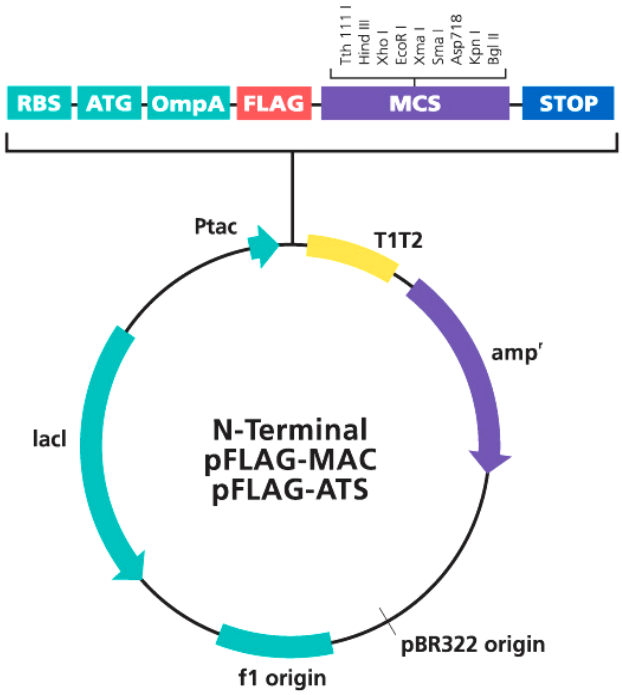


Figure 3.4 Map of expression vector pFLAG-ATS. It containing f1 and pBR322 origin of replication, Amp^R, *tac* promoter *lacI* repressor and *ompA* signal peptide from *E. coli*. The vector is specific for periplasmic protein expression in gram-negative bacteria like in *E. coli* BL21.

The active site residues of glutamate binding protein AatJ were replaced to alanine to probe their role in substrate binding. Arginine 71, S86, T87, T88, R93, R174 and double mutation of residues S86-T87, T87-T88, R71-R174 to alanine were produced by using set of primers as described in

section 2.3.4 (see materials). The mutated derivatives of vector pFLAG*aatJ* were finally transformed into *E. coli* BL21 cells for expression

E. coli cells with recombinant vectors were grown on 1 liter LB Amp medium at 37°C until the optical density A_{595} reaches to 0.8 to 1. Expression was induced by addition of 1 mM IPTG and additionally grown for 6 hrs at 37°C. The cells were harvested at 5000 rpm for 15 min at 4°C. Periplasmic fraction was separated according to protocol described by Harms et al, (Harms *et al.* 1991). In brief the cell pellets were resuspended in 200 ml ice cold spheroblast buffer (100 mM Tris-HCl, pH 7.8 with 0.5 M sucrose and 0.5 mM EDTA) and incubated for 20 min at 4°C. The cells were pelleted and resuspended in 20 ml ice cold milliQ water, incubated for 30 minutes at 4°C, followed by centrifugation at 18,000 rpm for 30 min at 4°C. Supernatant containing periplasmic fraction were separated by Q- sepharose™ anion exchanger column (GE Healthcare Biosciences) equilibrated with 50 mM Tris-HCl, pH 7.8 and eluted by linear gradient 0 to 500 mM of NaCl. The factions containing AatJ were pooled together and purified by gel filtration (Sephacryl-S100) high resolution preparative column (14x700 mM size) equilibrated with buffer (50 mM Tris-HCl, pH 7.8 and 100 mM NaCl). Purity of proteins was also judged by 15% SDS-PAGE. Pure fractions were pooled and concentrated using centricon (10,000 MWCO). Protein estimations were performed by BCA kit (Pierce) and stored at 4°C.

3.19 Electro mobility shift assay (EMSA)

3.19.1 Cloning of *aatJ* and *ansB* promoters

The upstream promoter region of the gene PP1071 (-83 bp to -343 bp relative to transcription start site) was originally amplified from *P. putida* KT2440 genome by using primers PromFor_PP1071 and PromRev_PP1071. The amplified fragment was cloned in pUC19 plasmid, sequenced and transformed to *E. coli* Top10 cells. The vector extracted from *E coli* was digested with EcoRI and HindIII. Purification of DNA was performed by agarose gel electrohporesis and extracted by using gel extraction kit (Quiagen). The promoter of gene *ansB* was amplified by using PromFor_PP2453 and PromRev_PP2453 the resultant fragment was purified by agarose gel. The amplification of different length promoter fragment for promoter deletion experiment of *aatJ*; the only forward primer PromFor_PP1071 and reverse primers PromRev_117, PromRev_136, PromRev_157, and PromRev_202 (summarized in section 2.3.3) were used. The PCR reaction was performed as; 95°C

initial denaturation for 4 min, 95°C denaturation for 1 min, 55°C annealing for 40 sec, 72°C extension for 40 sec and final extension at 72°C for 8 min until 35 cycles. The amplified fragments were purified by agarose gel.

3.19.2 Labelling of 5`-DNA end with ^{33}P

The DNA fragments were labeled with ^{33}P. The labeling reaction was carried out in 50 µl total reaction volume containing 10 µl of 5X forward reaction buffer, 5 pico moles of DNA fragment, 25U T4-Polynucleotide kinase (Stratagene) and 5 µl of γP^{33}-ATP (10µCi/µl, 110 TBq/mmol) the reaction mixture was incubated at 37°C for 1 hour. The labeled fragment was purified from unincorporated γP^{33}-ATP by using Microspin™ PSR-300 column (Amersham Biosciences). The specific activity was counted by scintillation. Unspecific fragment of about same size was also labeled by using same protocol.

3.19.3 Binding of promoter DNA with AauR

The DNA-AauR binding reaction was carried out as described by (Banerjee & Kundu 2003). Labelled DNA probe (5 fm) was added with 10 to 100 nM of AauR in HGMKE buffer (25 mM HEPES, pH 8 with 5% (v/v) glycerol, 10 mM $MgCl_2$, 20 mM KCl and 0.1 mM EDTA). The binding mixtures were incubated at 37°C for 20 min and separated in 6% native PAGE. Subsquently, the gel was dried, exposed to phosphor screen (Amersham Biosciences) for 24 hrs and scanned by phosphorimager (Molecular Dynamics). The binding specificity of AauR protein was also examined with unspecific DNA fragment of about same size using same protocol.

3.20 DNase I foot printing of *aatJ* promoter

DNase I foot printing was performed as described by (Licht & Brantl 2006). The labeled fragment was digested with BamH1 to delete 5`-^{33}P of one end and purified by phenol chloroform. Binding reactions were performed in a final volume of 20 µl containing HGMKE buffer, 0.05 g/l salmon sperm DNA, 50 ng labeled probe and 20 nm to 100 nm AauR. The reaction mixture were incubated at 37°C for 20 min and digested with 0.05U DNase I (Fermentas) for 2 minutes at 37°C. Digestion was stopped by extraction with phenol and subsequent precipitation with ethanol. The pellets were dissolved in 4 µl formamide loading dye and separated on 8% urea polyacrylamide sequencing gel

along with standard sequencing ladders (G+A and C+A) produced by Maxam-Gilbert chemical sequencing protocol as described in (section 3.10.4). The dried gel was exposed to phosphor screen for one week at room temperature and analyzed by phosphor imager (Molecular Dynamics).

3.21 *In-vitro* Phosphorylation of AauS proteins

Phosphorylation of sensor kinase (AauS) was performed *in-vitro* according to the protocol defined by (Rasmussen *et al.* 2005). Phosphorylation reaction was carried out in TGMNKD buffer (50 mM Tris-HCl, pH 8 with 10% (v/v) glycerol, 5 mM $MgCl_2$, 150 mM NaCl, 50 mM KCl and 1 mM DTT). For convenience 5× TGMNKD buffer was prepared and stored at -20°C, except 1M KCl and 1M $MgCl_2$ were prepared separately and added during reaction. In phosphorylation reaction 6 µM (200 µg) of AauS was added with 200 µl 1× TGMNKD buffer containing 50 mM freshly prepared cold ATP and 5 µl of γP^{33}-ATP (10µCi/µl, 110 TBq/mmol). The reaction mixture was incubated at 30°C for 2 hrs. Aliquots of 10 µl were taken out at different time interval and quenched in 5 µl of 3× SDS/EDTA loading dye (7.5% (w/v) SDS, 90 mM EDTA, 37.5 mM Tris-HCl (pH 6.8), 37.5% (v/v) glycerol and 0.3 mM DTT) and stored at -20°C if not analyzed immediately. The phosphorylated AauS was loaded on 12% SDS-PAGE mini gel and run at 150 volt, 20 mAmp for 1 hour at RT. The gel was dried at Gel dryer for 20 min at 80°C, exposed to phosphor screen for 24 hrs and analyzed by phosphor-imager.

3.22 Phosphate transfer to AauR by AauS

Phosphorylated sensor kinase (AauS) was investigated for the phosphotransferase activity to the response regulator (AauR). 200 µg AauS was phosphorylated for 30 min as described in section 3.21. Phosphorylation was performed by mixing equal quantity of phosphorylated AauS to AauR in TGMNKD buffer. Stability of phospho-AauR was studied by incubating the reaction mix for different times (0-60 min) and terminating the reaction by addition of SDS/EDTA loading dye. Concentration dependent experiments for phosphotransferase activity were also performed, in first set of experiments 1 µM aauR was taken and 0.25 to 10 µM phosphoralated AauS was added. In another set of experiment 1 µM AauS was taken with varying amount of AauR (0.25 to 10 µM). The reactions were quickly terminated by addition of SDS/EDTA loading dye and samples were

separated in 12% SDS-PAGE. The gel was dried, exposed to phosphor screen and analyzed by phosphor-imager.

3.23 Equilibrium micro-dialysis for protein-ligand binding

Equilibrium dialysis is one of the most commonly used methods to examine protein-ligand interaction at native and equilibrated state. In standard equilibrium dialysis, there are two chambers separated by dialysis membrane. A known concentration and volume of ligand placed into one of the chamber and another chamber is filled with receptor. The ligand is small enough to pass through the membrane. As ligand diffuses across membrane some of it will bind to receptor and some will remain free in the solution. The higher the affinity of the interaction, the higher the concentration of ligand that will be bound at any time. Diffusion of ligand across membrane continues until equilibrium has been reached.

At equilibrium concentration of ligands free in solution will be equal in both chambers. In the receptor chamber, however, the overall concentration is higher due to the bound ligand component. This type of assay typically performed using equilibrium dialysis falls under the category of saturation binding experiments. The relationship between binding and ligand concentration is then used to determine the number of binding sites, B_{max} and the ligand affinity.

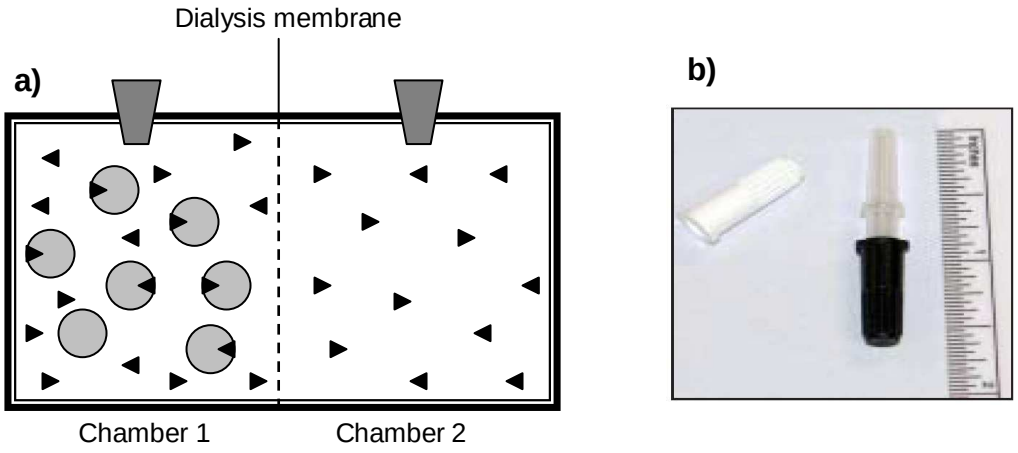


Figure 3.5 (a) Schematic representation of an equilibrium dialysis apparatus. Chamber 1 and 2 are separated by dialysis membrane. At the beginning chamber 1 is filled with receptor (protein) solution and chamber 2 is filled with buffer and ligand. After the equilibration reached, chamber 1 will represent the sum of free and bound ligand while chamber 2 will represent free ligands. **(b)** Equilibrium dialysis apparatus (Havard Apparatus, USA) is a disposable 25-100 µl volume capacity used in experiments.

3.24 Binding of AatJ and mutants with ^{14}C-Glu and ^{14}C-Asp

Purified AatJ was dialyzed against ligand binding buffer (50 mM HEPES, pH 7.5 and 100 mM NaCl) followed by concentrating to 10 µM using Centricon concentrator (10kDa MWCO, Millipore Inc). The binding reactions were carried out in ligand binding buffer with 50 µl of total reaction volume containing 5 µM AatJ or mutant protein. Ligands were used from 0.1 to 25 µM of ^{14}C labeled L-glutamate, L-aspartate (200 mci/mMol). In competitive binding experiments 10 fold unlabeled amino acids were used. Samples were loaded on one chamber of dispo-dialyzer (Havard Apparatus, Dispo-Equilibrium dialyzer) and another side was filled with buffer solution containing ligand, allowed to equilibrate for 4 hrs at 25°C by gentle rocking. The equilibration was monitored by control reaction. Equilibrated sample and buffer side solutions were added with 2 ml liquid scintillation cocktail and counted on scintillation counter. The fraction of bound ligand was estimated from the difference of both counts. As, in the present case, the ligand dissociation constants K_d^l are comparable to the concentration of the receptor (AatJ), ligand depletion had to be taken into account. In such cases, the concentration of the ligand-receptor complex, [RL], is given by

$$[RL] = \frac{([R]+[L]+K_d^l) - \mathrm{Sqrt}\{([R]+[L]+K_d^l)^2 - 4\,[R][L]\}}{2}$$

Where [R] and [L] are the total concentrations of receptor and ligand, respectively (Copeland 2000). Dissociation constants were estimated by fitting the above equation to the binding data. Binding constants K_d^a of unlabeled ligands (Asn, Gln and dicarboxylates) were estimated from their competitive effects on Glu and Asp binding, based on the equation

$$[RL] = \frac{[R] \cdot n}{1 + K_d^l/[L] \cdot (1 + [A]/K_d^a)}$$

Where n is the number of ligand binding sites in R and [A] is the total concentration of competing unlabeled ligand. The respective calculations were performed using the SigmaPlot 8.0 software (SPSS).

3.25 Native PAGE analysis of AatJ ligand binding

Purified AatJ and mutant proteins were dialyzed against ligand binding buffer (50 mM HEPES, pH 7.5 and 100 mM NaCl). 1 μM of AatJ and mutant protein was taken in 20 μl of ligand binding buffer and 0.1 to 5 μM ^{14}C labeled L-glutamate, L-aspartate (200 mci/mMol) were added. The samples were incubated at RT for 20 min. 5μl sample loading buffer was added to the samples and separated in 6% native PAGE as describe in section (3.11.1). The gel was dried at 85°C for 20 min, exposed to phosphor screen for overnight and analyzed by phosphorimager.

4. Results

4.1 Growth of *P. putida* KT2440 on amino acids

Many microorganisms use amino acids as alternative sources of carbon and nitrogen. Under conditions of nitrogen starvation, amino acids are preferably utilized. However, the efficiency of amino acid utilization varies widely among bacteria, so, for instance, *E. coli* is unable to utilize amino acids as sole nutrient or utilizes them poorly. In contrast, most amino acids are excellent growth substrates for *P. putida* KT2440, serving as the sole source of carbon and nitrogen or as nitrogen source.

Amino acids	*Pseudomonas putida* KT2440					
	amino acid			amino acid + glucose		
	ΔA (mAU)	μ_{max} (mAU/h)	lag (h)	ΔA (mAU)	μ_{max} (mAU/h)	lag (h)
Ala	-	<1	-	-	<1	-
Arg	830	77	8.3	1100	97	5.2
Asn	780	99	4.1	1220	94	3.3
Asp	820	105	6.2	1210	88	2.7
Cys	-	<1	-	-	<1	-
Gln	1140	103	5.4	1280	88	3.6
Glu	1150	100	6.8	1300	97	3.2
Gly	-	<1	-	1050	84	7.4
His	1070	82	5.9	1080	90	3.5
Leu	-	<1	-	-	<1	-
Lys	510	57	9.6	1020	92	7.9
Pro	1040	106	5.9	1140	97	4.1
Ser	-	<1	-	1130	101	6.7
Thr	-	<1	-	-	<1	-

Table 4.1: Growth characteristics of wild type *P. putida* KT2440 on 5 mM amino acids as sole source of carbon and nitrogen, or amino acids as nitrogen source supplemented with glucose as carbon source. The growth parameters shown in the table were estimated by fitting a modified logistics model (Zwietering *et al.* 1990) to the ΔA *vs.* t profiles. ΔA; final absorption change, μ_{max}; maximum rate of absorption change, lag; duration of the lag phase, -; no growth detected.

The growth properties of wild type *P. putida* KT2440 on selected amino acids are given in Table (4.1). Some of them (Ala, Cys, Leu and Thr) were unable to support growth. Gly and Ser were good sources of nitrogen in the presence of glucose as additional carbon source, while Glu, Asp, Gln, Asn, Arg, His, Pro and Lys supported rapid growth when supplied as sole source of carbon and nitrogen. Both the growth rates and final growth yields were highest during growth on the acidic amino acids (Glu, Asp) and their amides (Gln, Asn).

4.2 Growth properties of KT*aauR* mutants

The ability of *P. putida* KT2440 to utilize acidic amino acid depends on a functional *aau* system. In order to determine the role of *aau* two-component system, the gene *aauR* was knocked out by introducing a frame shift mutation.

Amino acids	KT*aauR* mutants					
	Amino acids			Amino acids+glucose		
	ΔA (mAU)	μ_{max} (mAU/h)	lag (h)	ΔA (mAU)	μ_{max} (mAU/h)	lag (h)
Ala	-	<1	-	-	<1	-
Arg	870	87	7.9	1010	108	5.2
Asn	770	130	7.1	1160	94	3.4
Asp	560	48	10.9	770	73	7.9
Cys	-	<1	-	-	<1	-
Gln	440	29	7.0	1150	95	4.1
Glu	470	65	14	860	78	9.8
Gly	-	<1	-	1020	87	6.2
His	1100	85	6.5	1080	92	4.0
Leu	-	<1	-	-	<1	-
Lys	580	60	8.7	900	92	7.4
Pro	1050	87	5.0	1060	107	4.9
Ser	370	29	10	1050	86	4.9
Thr	-	<1	-	-	<1	-

Table 4.2: Growth characteristics of *P. putida* (KT*aauR*) mutants on amino acids and amino acids supplemented with glucose. The growth parameters shown here, were estimated by fitting a modified logistic model (Zwietering *et al.* 1990) same as Table 4.1.

An internal region of *aauR* (300 bp) was amplified by inserting a single base for frame shifting and cloned into the pK18. The vector carrying the defective *aauR* fragment was electroporated into *P. putida* KT2440, yielding mutant KT*aauR*.

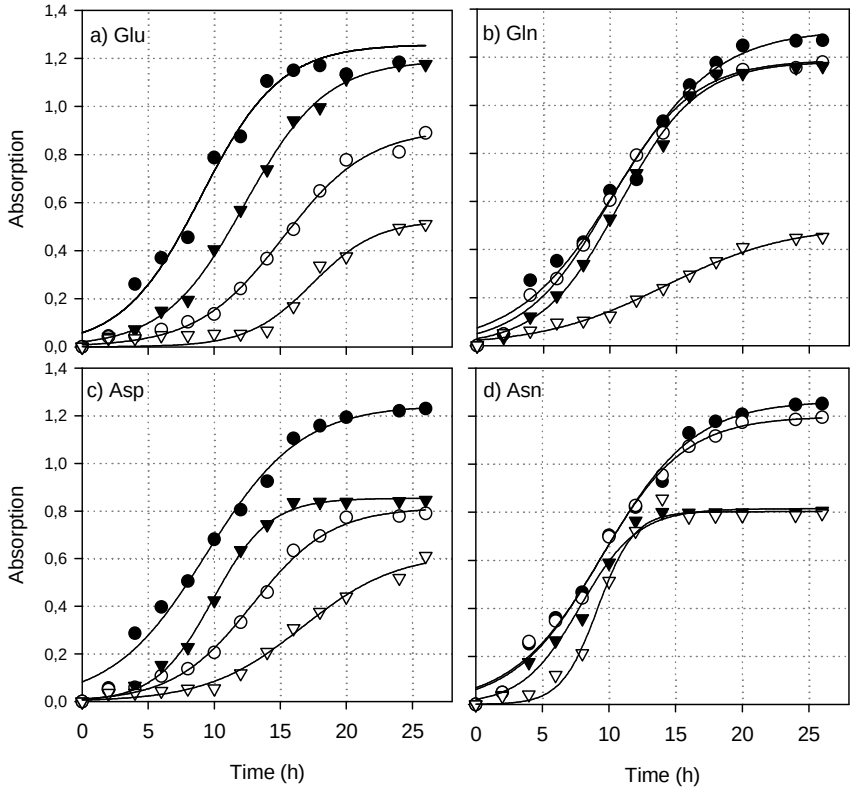


Figure 4.1: Growth curves of *P. putida* KT2440 (closed symbols) and KT*aauR* mutants (open symbols) on amino acids (triangles) and amino acids supplemented with glucose (circles). Cells were pre-grown overnight on $M9^+$ medium and then transferred to M9 minimal medium supplemented with 5 mM amino acid as sole source of carbon and nitrogen or glucose as carbon source. Growth rates were measured by absorption at 595 nm. Growth time *vs* change in A_{595} was plotted and fitted with a logistic model (Zwietering *et al.* 1990).

Then growth patterns were examined as defined for KT2440. Growth of the KT*aauR* mutant on Glu, Gln and Asp was strongly impaired when these amino acids were supplied as sole source of carbon and nitrogen. KT*aauR* did not proliferate at all for 10-15 h after transfer. In addition, the final cell densities attained after 24 h were significantly lower than with wild-type cells. Glucose supplied as additional source partially or fully restored the growth of KT*aauR* on Glu, Gln and Asp (Fig. 4.1). By contrast NH_4^+ supplied as second nitrogen source did not improve the growth of the mutant, whereas Asn or Asn/glucose allowed normal growth of KT*aauR*. Likewise, no significant

difference between growth rates of wild-type KT2440 and KT*aauR* were seen with most of the other amino acids tested with the exception of serine, which supported better growth of the mutant when given as the sole source of carbon and nitrogen.

4.3 Growth properties of mutants KT*aatJ* and *KTaatP*

Acidic amino acids and their amides are excellent sources of carbon and nitrogen for *P. putida* KT2440 (section 4.1), indicating that these amino acids can be efficiently imported for utilization. The dramatic effects of the gene *aat* deletion on growth rates (Table 4.3) imply that this system is indeed crucial for acidic amino acid uptake.

Amino acids	Wild-type KT2440			KT*aatP*			KT*aatJ*		
	A (mAU)	μ_{max} (mAu/h)	lag (h)	A (mAU)	μ_{max} (mAu/h)	lag (h)	A (mAU)	μ_{max} (mAu/h)	lag (h)
Glu	816	63.9	2.9	395	42.8	9.2	-	-	-
Gln	716	71.7	3.5	304	23.3	6.1	-	-	-
Asp	485	42.2	2.3	361	39.2	3.4	347	34.1	6.2
Asn	524	57.3	1.2	390	76.7	4.7	385	35.0	3.0

Table 4.3: Growth characteristics of wild type *P. putida* KT2440 and KT*aatP* and KT*aatJ* disruptive mutant grown on M9 medium supplemented with acidic amino acids as sole source of carbon and nitrogen. The growth parameters were estimated by fitting a modified logistics model ((Zwietering *et al.* 1990)) to the A *vs.* t profiles. A; final absorption, t; time, μ_{max}; maximum absorption change in the logarithmic phase, lag; duration of the lag phase, -; no growth detected.

Mutants of the solute binding protein gene (*aatJ)* showed highly impaired growth. Growth of KT*aatJ* was not detectable when Glu and Gln were supplied as the sole source of carbon and nitrogen (Fig. 4.2a and b) however, growth was partially retained when glucose was supplied as additional carbon source. Growth of KT*aatJ* on Asp and Asn was faster but the lag phases were almost doubled and cell yields were reduced by 50% (Fig. 4.2c and d). Growth of strain KT*aatP* (lacking a functional nucleotide-binding domain) on Glu and Asp was comparably better while growth of this mutant on Asp and Asn was normal (cf. Table 4.3). The growth profiles of strain KT*aatP* on Glu and Gln were characterized by prolonged lag phases in (>9 h and >6h, respectively as compared to 2.9 h and 3.5 h in the wild-type) while cells yields were almost half of those with wild-type KT2440. The growth properties of both mutants on other amino acids were the same as with wild type *P. putida* KT2440 (data not shown). Glucose and NH_4^+ as carbon and nitrogen

source also supported normal growth of the mutants. These data clearly show that the ABC transporter encoded by operon *aatJMQP* is essential for growth on acidic amino acids as sole source of carbon and nitrogen.

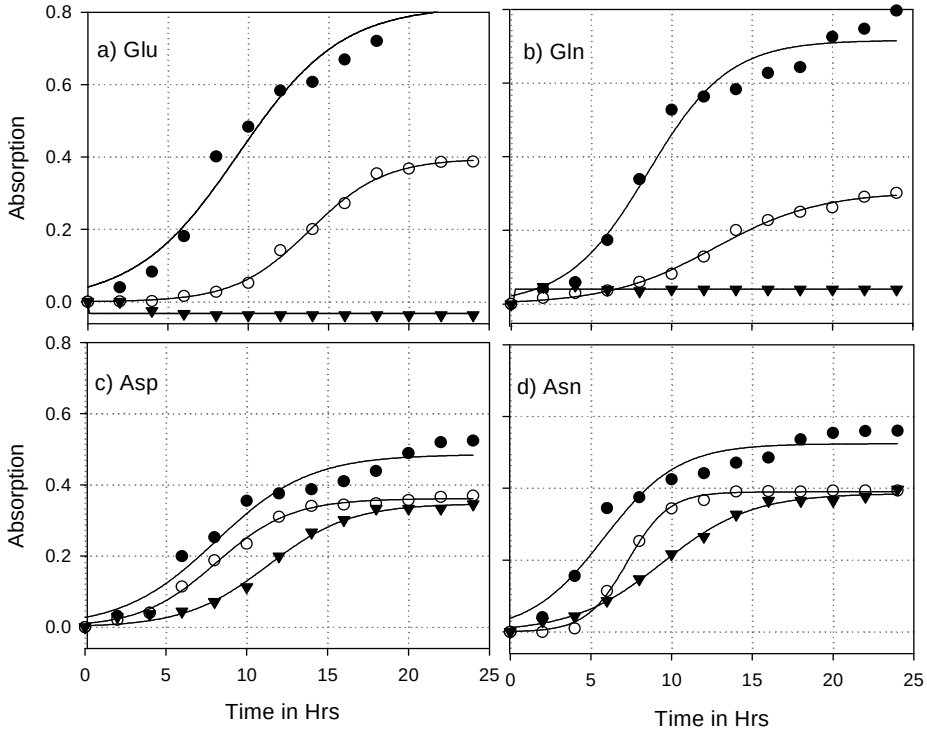


Figure 4.2: Growth of *Pseudomonas putida* KT2440 (●), mutant KT*aatP* (○) and mutant KT*aatJ* (▼) on amino acids. Cells were grown overnight on $M9^+$ medium (see 'Methods') and then transferred to M9 minimal medium supplemented with 5 mM amino acids as sole source of carbon and nitrogen. Growth rates were estimated from absorption measurement at 595 nm between 0 to 24 h. The solid lines are fits of a modified logistic model to the data (Zwietering *et al.* 1990).

4.4 Amino acid utilization by KT*aauR* mutants

In order to examine whether the AauRS system also controls amino acid transport, we examined the uptake of acidic amino acids and their amides by wild-type *P. putida* KT2440 and the KT*aauR* mutant. After the transfer of cells pre-grown on $M9^+$ medium to amino acid-containing medium, the concentrations of the respective amino acids in the medium were monitored as a function of time by quantitative amino acid analysis.

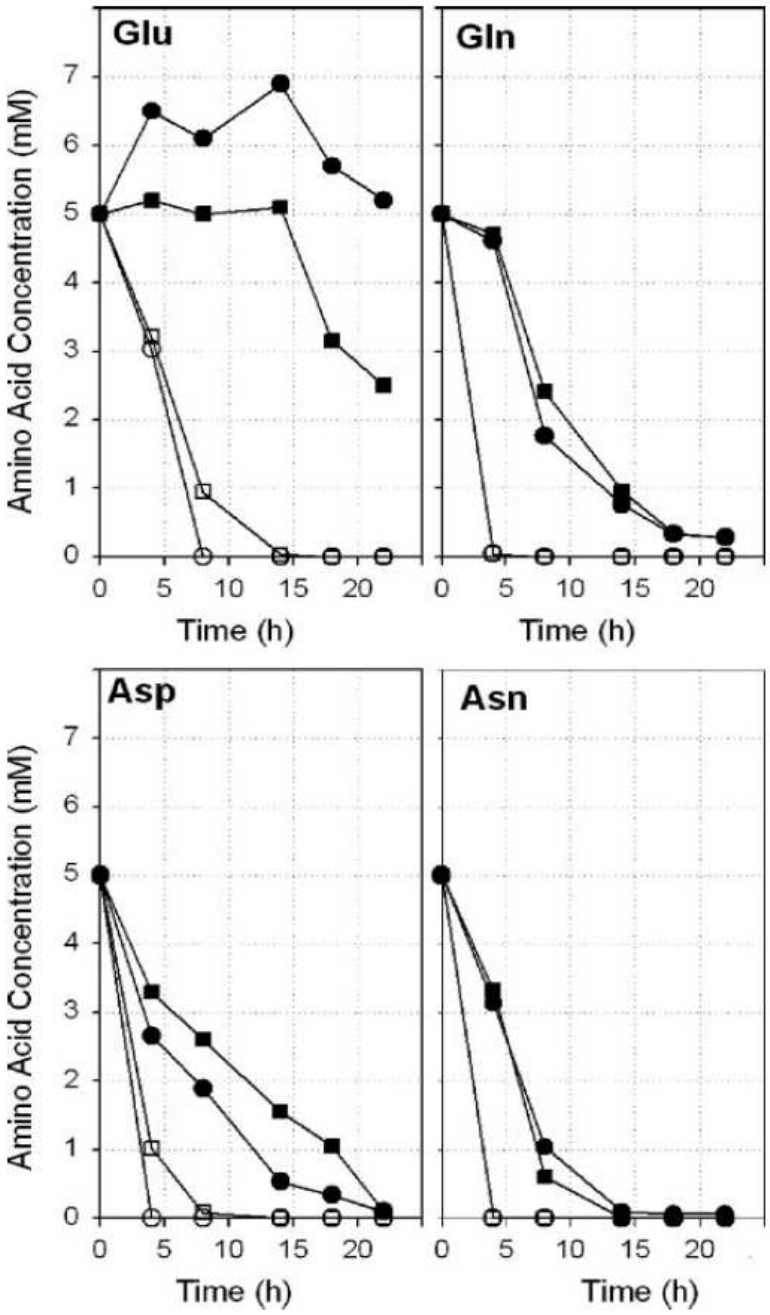


Figure 4.3: Amino acid uptake by *P. putida* KT2440 (open symbols) and the KT*aauR* mutant (closed symbols) during growth on 5 mM amino acids (circles) and on 22 mM glucose with amino acids (squares). The cells were pre-grown on $M9^+$ medium (see 'Methods') overnight and then transferred to M9 minimal medium supplemented with 5 mM amino acids as the sole carbon and nitrogen sources and amino acids in combination with 22 mM glucose. High initial cell densities (A_{595} = 0.5 to 0.6) were used for these experiments. The concentrations of the amino acids remaining in the medium are shown as a function of incubation time.

As shown in Fig. 4.3, the wild-type strain consumed all of the amino acids tested within 5 to 8 h after transfer (note that in these experiments the initial cell densities were markedly higher than those in Fig. 4.1). In the KT*aauR* deletion mutant, the consumption of Gln, Asp, and Asn was moderately delayed, whereas Glu was not taken up at all until growth of the mutant commenced after 15 to 20 h. In the absence of glucose, the mutant cells even secreted glutamate into the medium, as indicated by an increase of the initial Glu concentration by about 30%.

4.5 Amino acid transport by mutants KT*aatP* and KT*aatJ*

In order to study acidic amino acid uptake by the Aat ABC transporter, the cells were grown on Glu and Asp as the sole sources of carbon and nitrogen. The transport capacity of cells for uptake of ^{14}C-labelled Glu and Asp in the logarithmic phase was then measured by a quick filter assay.

The kinetics of transport by *P. putida* KT2440, KT*aatJ* and KT*aatP* (normalized with respect to total cellular protein) are shown in Fig. 4.4a. In both mutants, uptake rates were reduced to 10-20% of the wild-type values. The selectivity of uptake was verified by adding 50-fold excess cold amino acids which reduced uptake of the respective labeled amino acids by more than 80%, while Gln and Asn did not compete significantly. This kind of competitive uptake could not be performed with the mutants due to the slower rate of uptake. The uptake rates at higher amino acid concentrations were determined by HPLC analysis, where consumption of amino acids per ml of medium was calculated within a time interval (h), as shown in Fig 4.4b, the wild-type cells consume 5 mM of amino acids (Glu/Asp) within 6-8 hrs, however, uptake by KT*aatJ* and KT*aatP* mutants gradually slowed down and ceased after 6 hrs. The uptake rates determined by both these approaches were roughly equivalent with highly impaired uptake rates of KT*aatJ* and KT*aatP* mutants.

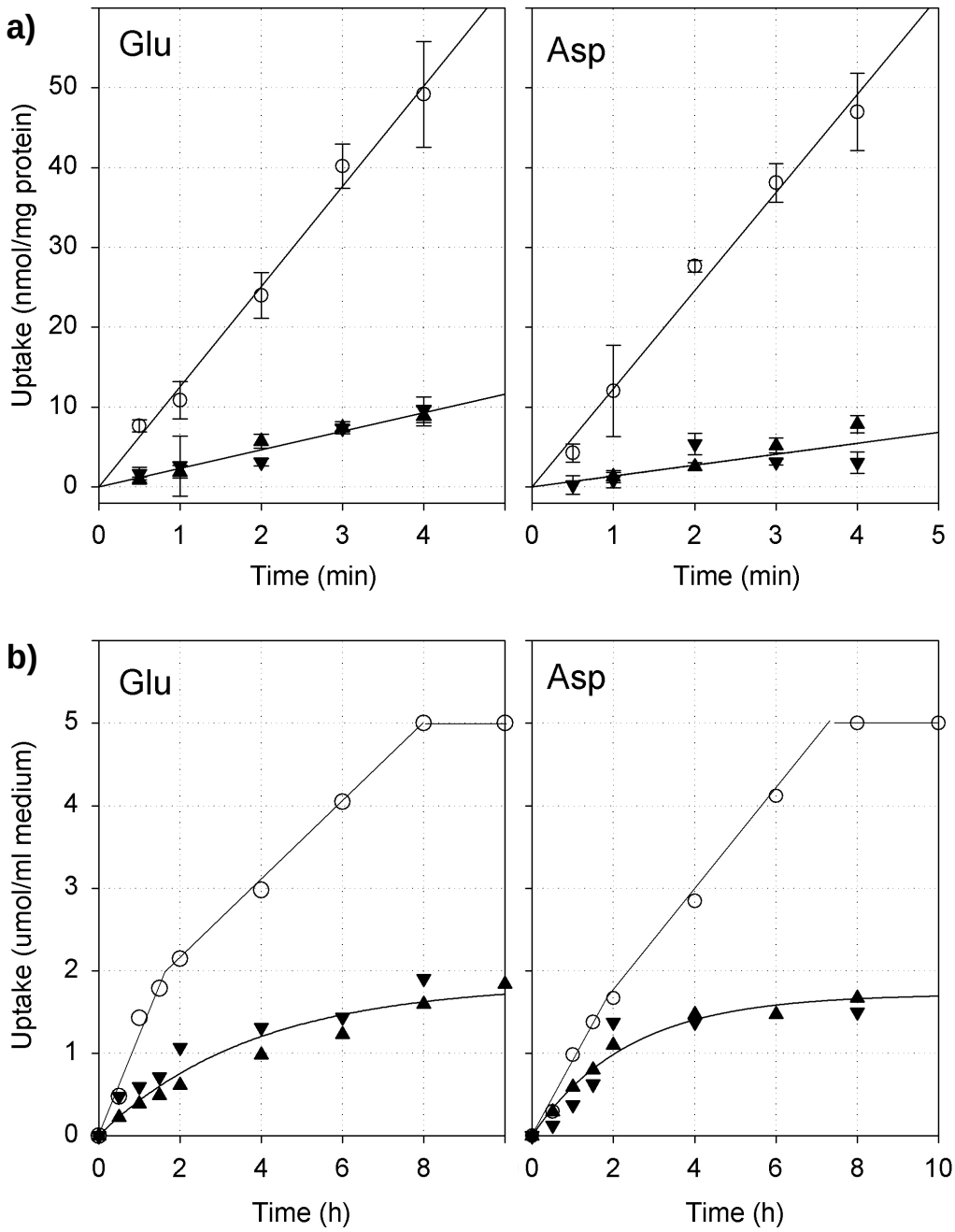


Figure 4.4: Kinetics of Glu and Asp uptake by *P. putida* KT2440 (○), mutants KT*aaJ* (▲) and KT*aatP* (▼) at low and high amino acid concentrations. **(a)** ^{14}C labeled L-Glu and L-Asp were added at zero time to a final concentration of 5 μM. Aliquots were removed from cultures at 30 s intervals, mixed up with stop solution and filtered through nitrocellulose membrane. The washed and dried filters were analyzed by scintillation counting. **(b)** After adding unlabeled Glu and Asp to a final concentration of 5 mM at zero time, the decrease of amino acid concentration in the medium was followed by HPLC analysis. Cumulative uptake is expressed as μmol of amino acid consumed per ml of medium.

4.6 Physiological analysis of KT*aauR* mutants

AauR is a σ^{54}-dependent response regulator of the AauR/S two-component system. This kind of regulator acts as an additional transcription factor of RNA polymerase during transcription of bacterial genes. As mutants defective in *aauR* (KT*aauR*) were unable to grow on acidic amino acids and were also defective in uptake of these amino acids, we further studied enzymes involved in the metabolism of acidic amino acids. *P. putida* shares many common ammonia assimilation pathways with enteric bacteria. These involve the GS/GOGAT and GDH pathways for consumption of acidic amino acids (cf. 'Introduction' for details).

4.6.1 Expression of periplasmic glutaminase/asparaginase (PGA)

PGA is an amidohydrolase that converts Gln and Asn to the respective acidic amino acids, releasing one ammonium ion. According to their sub-cellular localization and kinetic properties, there are two subgroups of asparaginases. Class I asparaginases are cytoplasmic and constitutive enzymes, while class II are located in the periplasmic space and hydrolyze both Gln and Asn to allow uptake of Glu and Asp. Glutamine as such cannot be transported by *P. putida*, as PGA mutants do not grow on Gln as the sole source of carbon and nitrogen while they grow well on Glu (Sonawane, 2003a).

As shown in Fig. 4.5, the activity of PGA in *P. putida* KT2440 is completely under the control of the *aau* system. The PGA activity in KT*aauR* mutants was reduced 20-50 fold in comparison to wild-type KT2440 and was even undetectable in some cases. The highest PGA activity was found in wild-type cells grown on Asn and Asp. In cells growing on glutamate and glutamine PGA induction was delayed and only detected after 10 h, while PGA activity appeared after 2-3 h on Asp and Asn. Glucose as the carbon source reduced PGA activity 7-10 fold due to carbon catabolite repression.

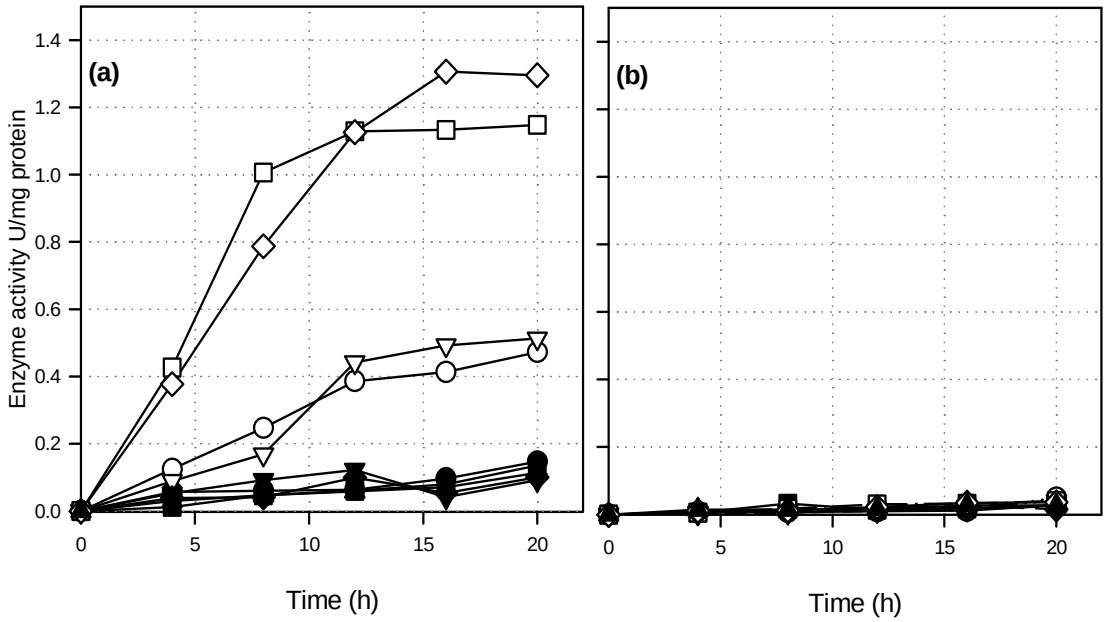


Figure 4.5: PGA activity of *P. putida* KT2440 **(a)** and mutant KT*aauR* **(b)** on 5 mM amino acids (open symbols) or 5 mM amino acids + 22 mM glucose (closed symbols). o,● - Glu, ▽,▼ - Asp, □,■ - Gln, ◊,◆ - Asn, ▲- 10 mM NH_4^+ + 22 mM glucose.

4.6.2 Expression of glutamate synthase (GOGAT)

Glutamate synthase (GOGAT) is one of the most important enzymes in ammonia assimilation by Gram-negative bacteria. The enzyme produces two molecules of glutamate from 2-oxoglutarate and glutamine. In our experiments, Fig. 4.6, the GOGAT activities in mutant KT*aauR* were considerably higher than in wild-type KT2440 (0.1 to 0.3 U/mg protein as compared to 0.02 to 0.1U/mg in the wild type).

4.6.3 Expression of glutamate dehydrogenase (GDH)

Glutamate dehydrogenase is usually most active at high levels of ammonia where it incorporates into 2-oxoglutarate to yield glutamate (see 'Introduction'). In comparison to wild-type KT2440, the GDH activity in mutant KT*aauR* was 10 to 20 times higher (Fig. 4.6). When KT*aauR* was grown on Gln as the sole source of carbon and nitrogen, maximum activities 0.3 to 0.35 U/mg of cellular protein were observed. During growth on NH_4^+/glucose there was no difference in GDH activities

between mutant and wild-type cells. Glucose as a carbon source did not exert significant catabolite repression on GDH.

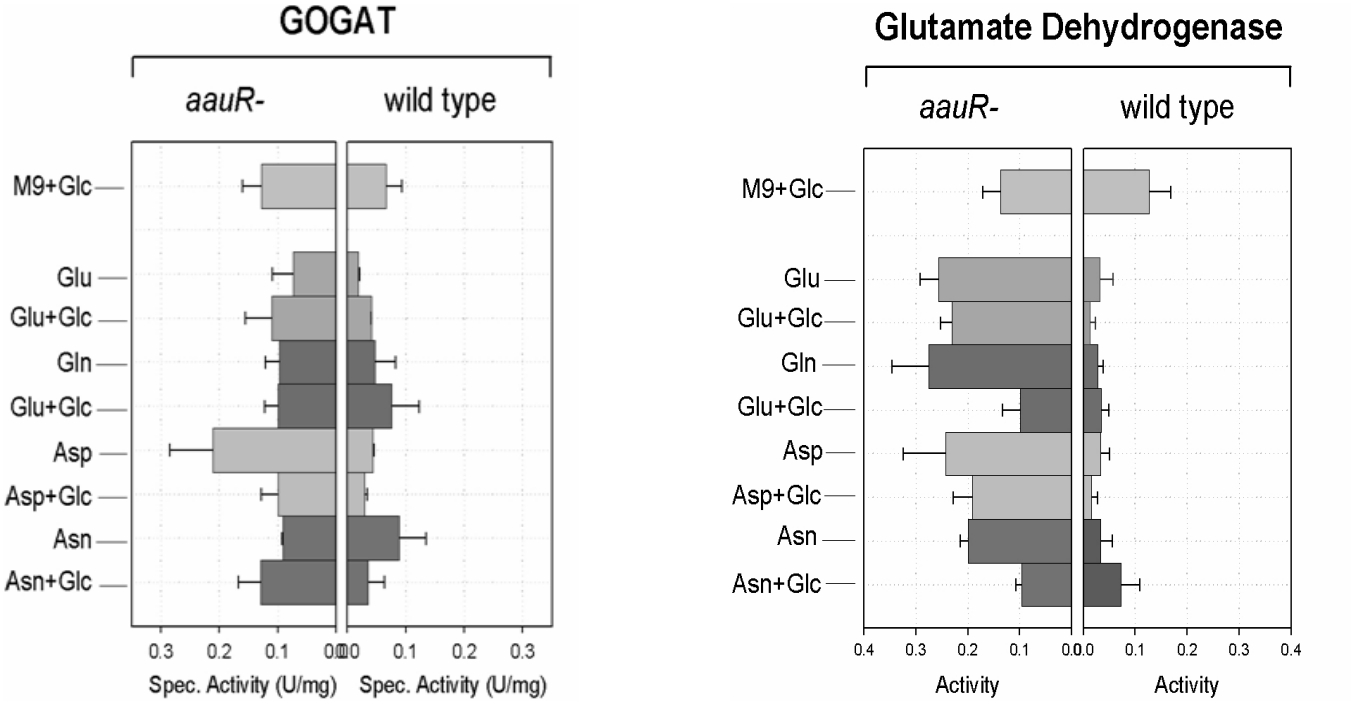


Figure 4.6: GOGAT and GDH activities of *P. putida* KT2440 and mutants KT*aauR* during growth on acidic amino acids. 5 mM amino acids were supplied as sole carbon and nitrogen source (aa) or together with or 22 mM glucose (aa+Glc). Bars represent standard deviation.

4.6.4 Expression of glutamine synthetase (GS)

Glutamine synthetase is a highly regulated enzyme which controls the level of glutamine and glutamate inside cells (see 'Introduction'). *P. putida* KT2440 and KT*aauR* mutants did not exhibit any significant difference in the GS activity (cf. Fig. 4.7).

4.6.5 Expression of aspartate lyase (aspartase)

Aspartase converts aspartate into fumarate and ammonia and thus feeds fumarate to the central metabolic pathways. Mutant KT*aauR* showed much higher aspartase activities than wild-type cells (0.2U to 1.5 U/mg of cellular protein as compared to 0.002-0.2 U/mg in the wild type (Fig. 4.7). Maximum activity of aspartase was found in KT*aauR* cells growing on Gln and Asp. When glucose was supplied as additional carbon source, aspartase activity was repressed due to carbon catabolic repression.

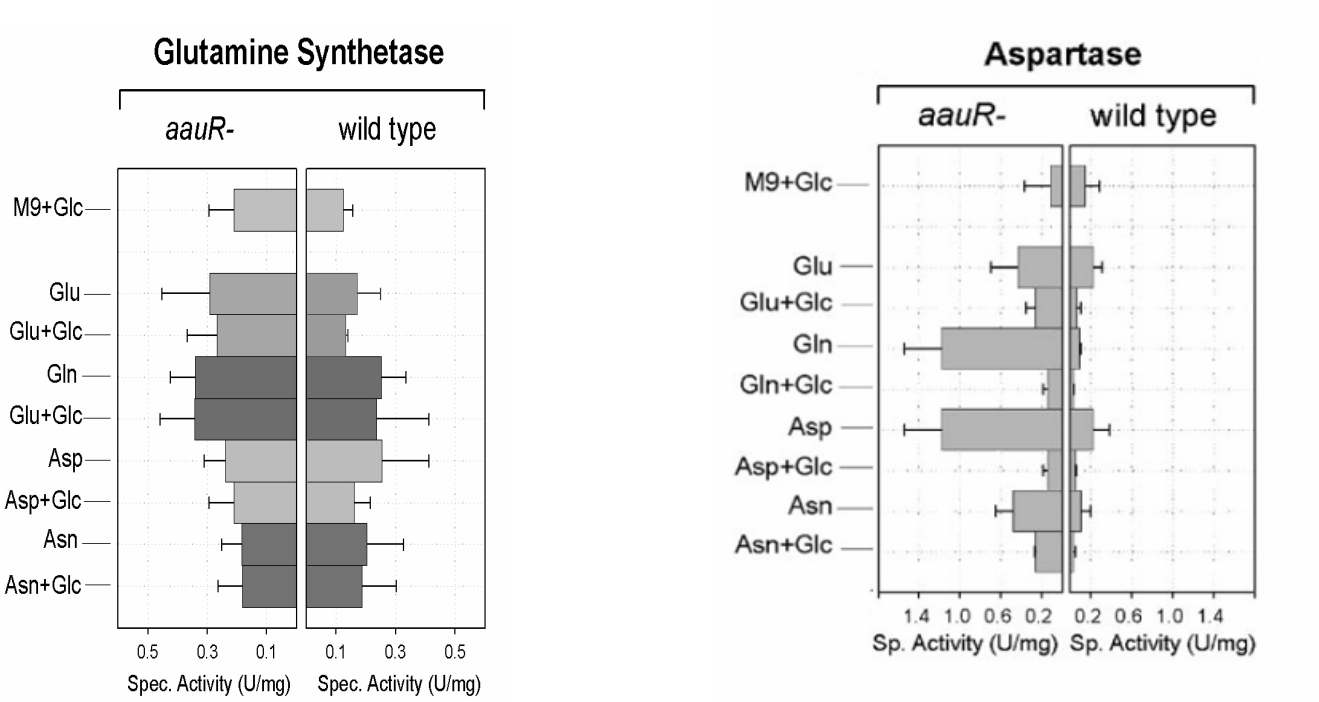


Figure 4.7: Glutamine synthetase and aspartase activities in *P. putida* KT2440 and mutant KT*aauR*. 5 mM amino acids were supplied as sole source of carbon and nitrogen source (aa) or together with 22 mM glucose (aa+Glc). Bars represent standard deviation

4.7 Determination of intracellular pools of glutamate and aspartate

In many microorganisms the intracellular pools of glutamate and aspartate are tightly controlled. In order to determine the importance of the AauRS system for the metabolism of Glu and Asp, their intracellular levels were measured in the late log phase. The intracellular concentrations of aspartate were same in the wild type and KT*aauR* cells while, the glutamate level was significantly different. In the mutant strain KT*aauR*, Glu accumulated in the late log phase. This effect was especially significant when the mutants were grown in Asp or Asn as sole source of carbon and nitrogen (cf. Fig. 4.8).

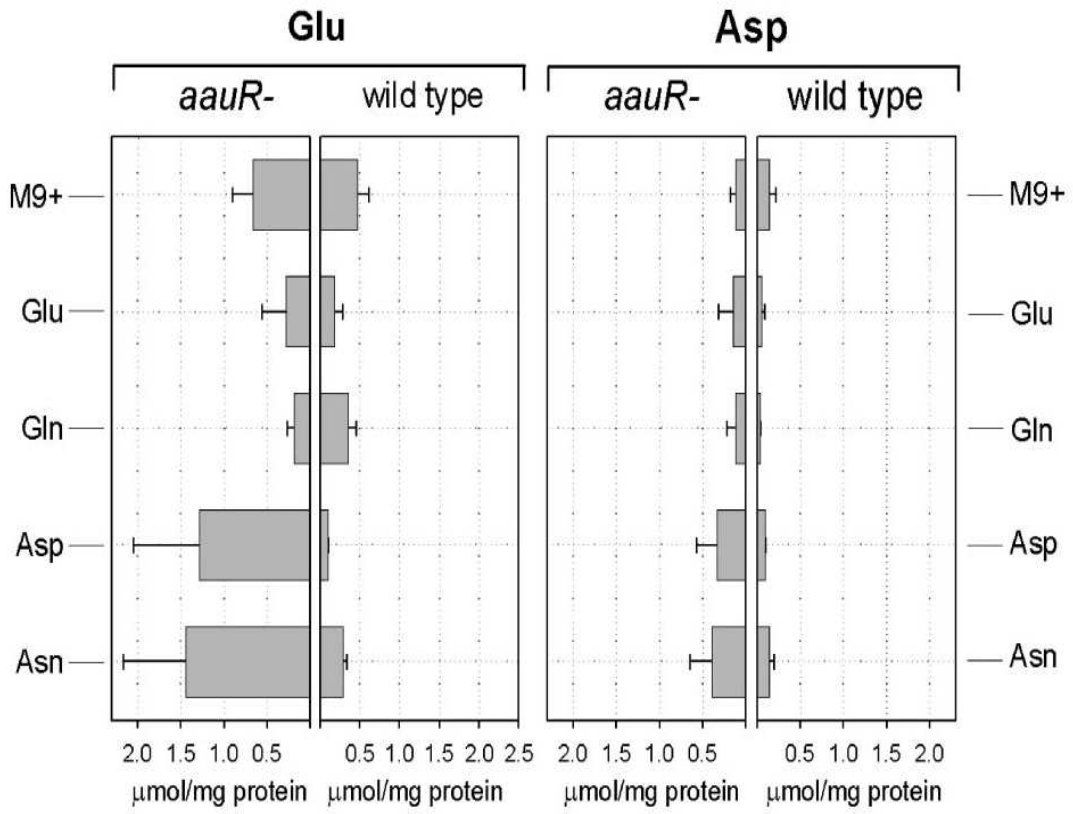


Figure 4.8: Intracellular concentrations of glutamate and aspartate (expressed as µmol per mg of cellular protein) after transfer to M9 minimal medium containing 10 mM each of Glu, Gln, Asp, and Asn. The error bars indicate standard deviations.

4.8 Survival of mutant KT*aauR* under conditions of nitrogen starvation

P. putida mutants deficient in nitrogen regulation were shown to be impaired in their ability to survive under conditions of nitrogen limitation. Mutant KT*aauR* showed the same kind of survival pattern when incubated for several days with acidic amino acids as sole source of carbon and nitrogen. As shown in Fig. 4.9, wild-type cells could maintain their viability for more than 16 days, whereas the KTaauR mutant cell count dropped steeply and after 6 days less than 1 % of the cells initially present had survived. This finding strongly suggested that AauR is indispensable for the survival of *P. putida* on Glu and Asp.

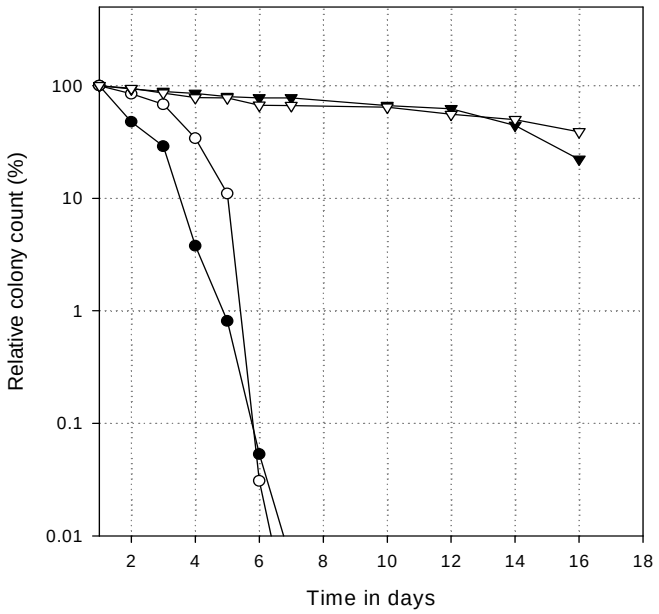


Figure 4.9: Starvation survival curve (viable cell count) of *P. putida* KT2440 (triangles) and cells KT*aauR* mutants (spheres). Filled signs show survival in glutamate and blank in aspartate. The experiment was performed as described in section 3.5.

4.9 Expression and purification of variants AatJ and AatJ

The Aat ABC transporter of *P. putida* is the product of four genes (PP1068-PP1071). Gene PP1068 codes for the ATP-binding cytosolic domain (AatP), genes PP1069-PP1070 encode the membrane-spanning permease domains (AatM and AatQ), and PP1071 encodes for the periplasmic solute-binding protein (AatJ). All these proteins are monomeric except the nucleotide binding domain AatP which is dimeric. The role of AatJMQP in the uptake of acidic amino acids was determined by amino acid uptake assay in mutants of AatJ and AatP (c.f. section 4.5). The binding properties of AatJ for acidic amino acids and other dicarboxylates were determined after expression of AatJ in *E. coli* BL21 cells and purification of the recombinant protein. In addition, the site directed mutagenesis approach was used to construct several point mutants of AatJ with the aim to identify residues involved in ligand binding.

The gene encoding AatJ (PP1071) is 918 bp long, corresponding to a 33.4 kDa protein. The first 72 bases which encode the signal peptide for export into the periplasm were deleted when cloning *aatJ* in the expression plasmid pFLAG-ATS (Sigma). This vector contains the *tac* promoter with an *Omp*A signal and a small flag sequence for transport of the recombinant protein

into the periplasmic space (cf. Fig. 3.4). The over-expressed protein was successfully exported, as the periplasmic fraction contained large amounts of recombinant AatJ (Fig. 4.10b). The final yields of purified AatJ were 15-20 mg/l of culture with a purity of more than 95%. The expressed AatJ was monomeric as indicated by analytic gel filtration.

Mutations of the *aatJ* gene were performed on the pFLAG-*aatJ* template using the Quickchange site directed mutagenesis kit as described in 'Methods' (section 3.18). The mutant vectors were sequenced and individually transformed into *E. coli* BL21. The expression and purification protocol was the same for wild-type AatJ and all the mutants.

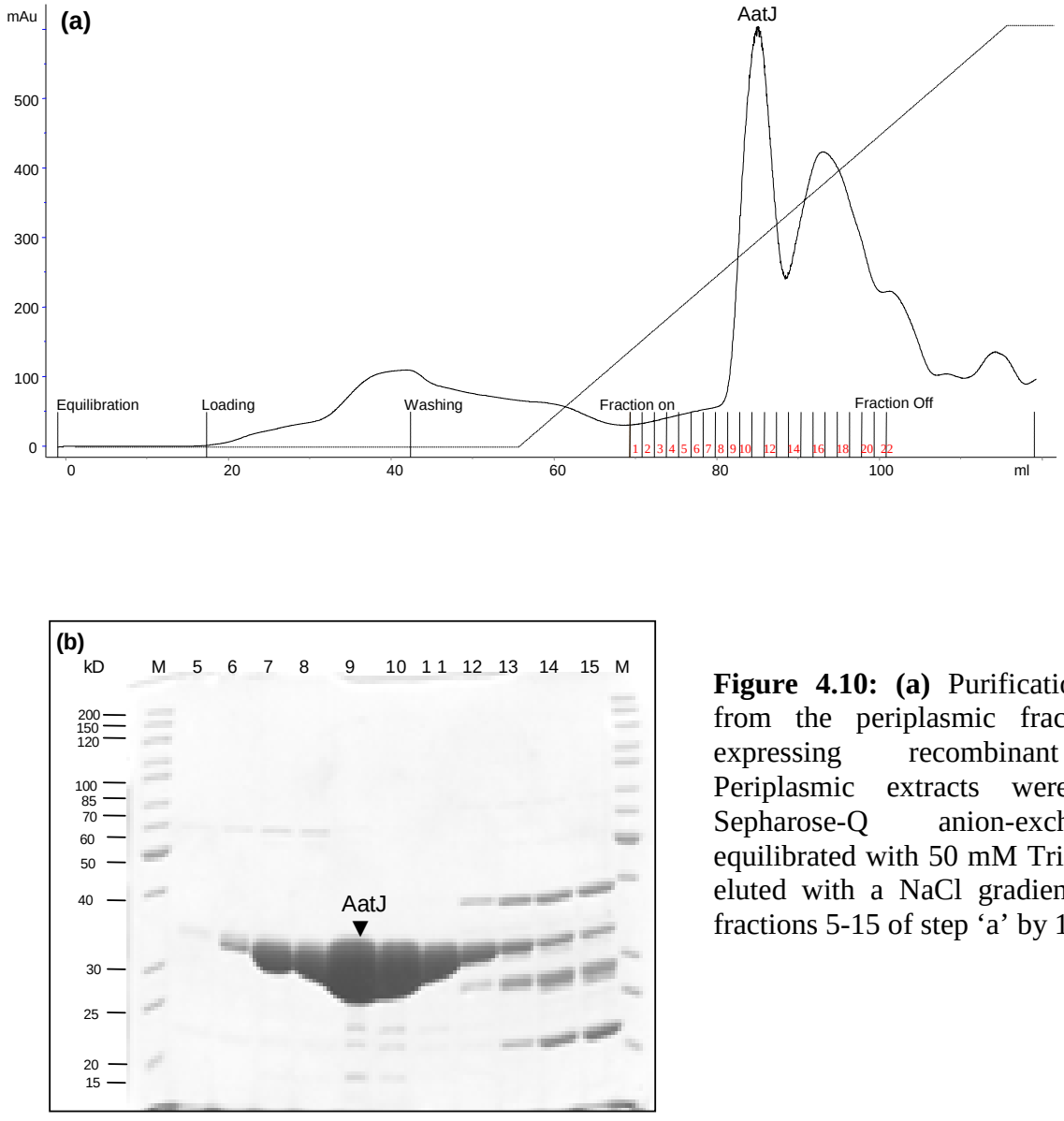


Figure 4.10: (a) Purification of AatJ (WT) from the periplasmic fraction of *E. coli*, expressing recombinant pFLAG-*aatJ*. Periplasmic extracts were loaded on a Sepharose-Q anion-exchange column equilibrated with 50 mM Tris-HCl, pH 7.8 and eluted with a NaCl gradient **(b)** Analysis of fractions 5-15 of step 'a' by 12% SDS-PAGE.

4.10 Binding of Glu and Asp to wild-type AatJ and mutants

For quantitative assays of amino acid binding to AatJ, we chose the equilibrium dialysis technique. When radioactively labeled ligands are available, this approach is simple and reliable and allows the estimation of true thermodynamic dissociation constants down to the μM and nM range. The saturation curves for Glu and Asp binding to AatJ are depicted in Fig 4.11 (a semilogarithmic plot is shown for clarity). As the receptor concentration is in the same range as the dissociation constant of the receptor/amino acid complex, ligand depletion had to be taken into account (see 'Methods'). Fig. 4.11 shows that our equilibrium binding data could be satisfactorily fitted by such a model. The calculated dissociation constants were 0.3 μM for Glu and 1.5-2 μM for Asp.

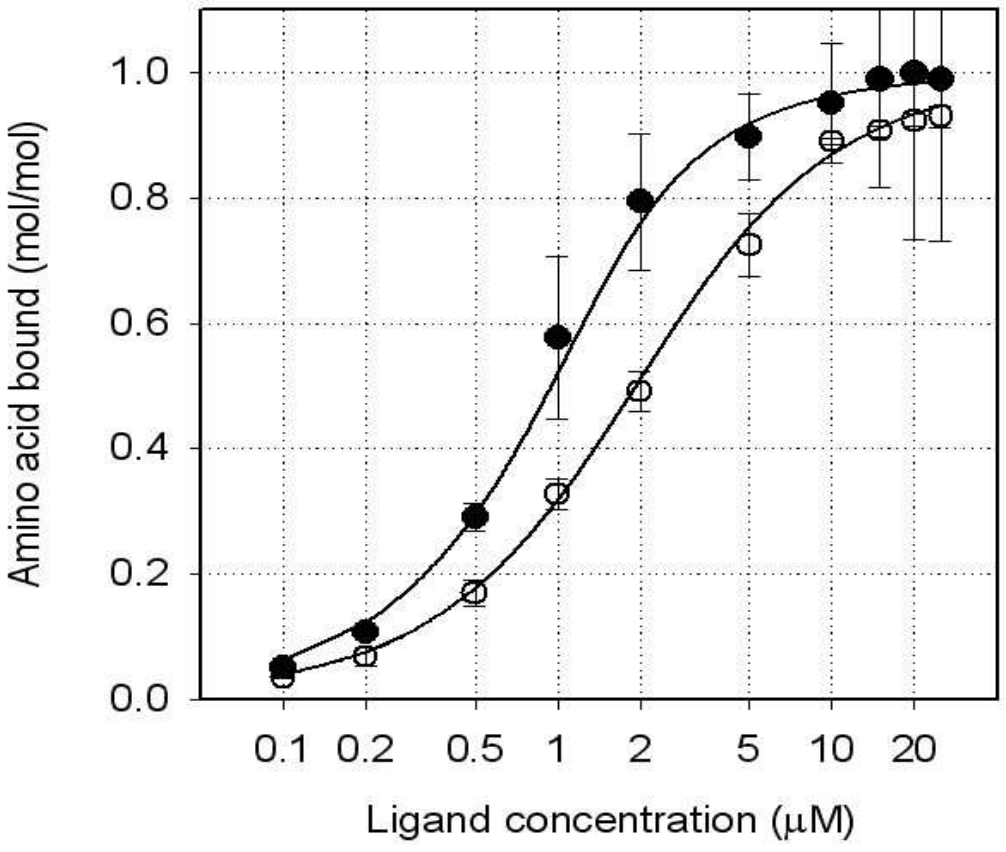


Figure 4.11: Binding of ^{14}C-Glu (●) and ^{14}C-Asp (○) to AatJ. Binding curves of fractional saturation (mol ligand bound/mol monomeric protein) *vs* the logarithm of total ligand concentration are shown. Solid lines represent best fits of the model equation employed (see method). Mean values ± standard deviation of three experiments are shown.

4.11 Competitive ligand binding

Since the amides Gln and Asn were not available in a radiolabeled form, a competition method was applied to determine their binding characteristics. In this approach, the reduction in binding of labeled ligand in the presence of a large excess of unlabeled amino acid is used to estimate the dissociation constant of the competing unlabeled ligand. The dissociation constants calculated in this way from data obtained with various combinations of labeled and unlabeled ligands are summarized in Table 4.4. In general, the values obtained by direct binding and competition experiments, respectively, are in good agreement except for aspartate, where the competition data indicate somewhat higher dissociation constants than the direct assay. While the affinity of AatJ towards asparagine is comparable to that observed with Asp, glutamine and the dicarboxylates fumarate and succinate were very weak ligands with dissociation constants too high to be estimated with certainty.

Compound	**K_d (μmol/L)**	**Estimated from**
L-Glutamate	0.3 ± 0.1 0.4 0.3	ED C_{Glu} C_{Asp}
L-Aspartate	1.5 ± 0.2 7 9	ED C_{Glu} C_{Asp}
L-Glutamine	>100 80	C_{Glu} C_{Asp}
L-Asparagine	18 15	C_{Glu} C_{Asp}
Succinate	> 100 > 100	C_{Glu} C_{Asp}
Fumarate	>100 >100	C_{Glu} C_{Asp}

Table 4.4: Calculated dissociation constants K_d of AatJ ligand complexes. ED; determined by equilibrium dialysis with ^{14}C labeled ligand. C_{Glu}- estimated from inhibitory effect on Glu binding. C_{Asp}- estimated from inhibitory effect on Asp binding.

The binding of ^{14}C-Glu and ^{14}C-Asp were also determined by semi-quantitative method using native-PAGE. 1 μM of AatJ was incubated with various concentrations of radio-labeled Glu and Asp, and then analyzed by native PAGE, followed by autoradiography. Competitive binding was studied by adding 50 μM cold amino acids to the samples (Fig. 4.12). Binding of Glu to AatJ was already seen after incubation with 0.1 μM ligand, while binding of detectable amounts of ^{14}C-Asp required ligand concentrations above 1μM, which indicates a 5-10 times lower affinity of AatJ toward Asp.

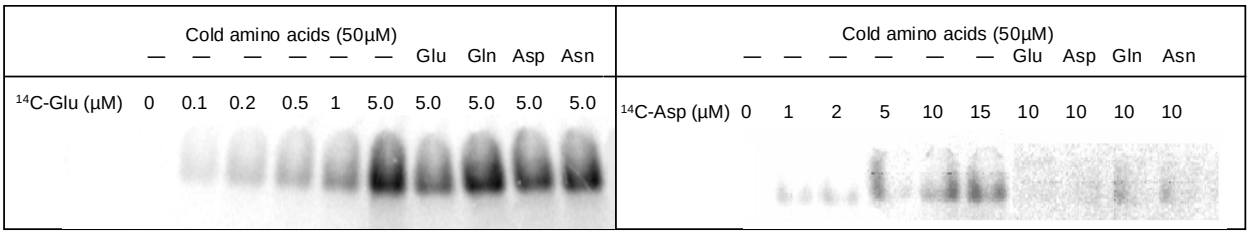


Figure 4.12: Binding of glutamate and aspartate to AatJ (WT). 1 μM purified AatJ was incubated with ^{14}C-Glu (0.1-5 μM) or ^{14}C-Asp (1-15 μM) in 50 mM HEPES buffer, pH 7.8 containing 100 mM NaCl. Competitive binding was performed by adding 50 μM cold amino acids together with 5 μM labeled Glu or 10 μM labeled Asp. Ligand binding was detected by autoradiography following 5% native PAGE.

4.12 Homology modeling of AatJ

Homology modeling of proteins is a modern *in-silico* approach where protein templates of known structure are used to model structures of an unknown protein. This approach is especially useful when the crystallization of a protein is difficult. However, the models generated by homology modeling should be validated by experimental approaches like site directed mutagenesis. According to its functional characteristics and sequence homology, AatJ belongs to the solute-binding protein family 3. This protein family includes some proteins of known crystal structure like GlnH of *E. coli,* Peb1a protein of *Campylobacter jejuni*, HisJ and the arginine/ornithine/lysine-binding protein (LAOBP) of *E. coli* and *Salmonella typhimurium.*

In order to construct a homology model of the AatJ/glutamate complex, we selected the closed conformation of the *E. coli* glutamine binding protein GlnH (PDB entry 1wdn) as the

template. In a first step, the Swiss Model server (Schwede *et al.* 2003) was utilized to model the closed form of unliganded AatJ. Then the ligand (Glu) was inserted into the model roughly at the position where Gln resides in GlnH, followed by a 10 ps molecular dynamics simulation of the resulting complex, using the Amber99 force field (Cornell *et al.* 1995) as implemented in the HyperChem software package (Hypercube, Inc.). During the simulation, an ensemble of ligand conformations remained relatively stable over several picoseconds. One of these structures was selected and energy-minimized with Amber99 to yield the final model. The environment of the bound glutamate in this modeled structure is shown in Fig. 4.13. Clearly, the overall binding mode of the ligand is similar to those seen in the experimental structures, i.e. R93 and T88 (possibly S85 as well) interact with the α-carboxylate and α-ammonium group of the bound amino acid. Interestingly, in the modeled structure the γ-carboxylate of the ligand is positioned between two further arginine residues, R71 and R174, with R71 occupying the more favorable position. This result is obviously consistent with the observed preference of AatJ for acidic amino acids.

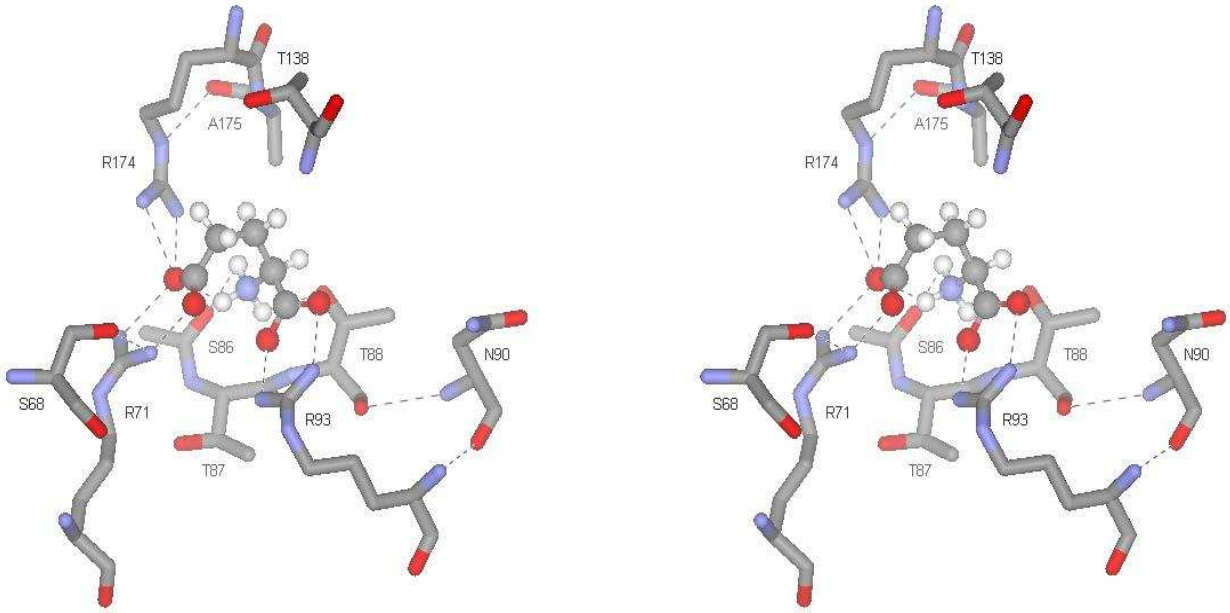


Figure 4.13: Stereo view of the modeled amino acid binding site of AatJ. Bound glutamate is shown in ball-and-stick representation. The numbering of the amino acid residues excludes the signal peptide of the AatJ precursor which has a length of 24 residues as predicted by PrediSi (Hiller *et al.* 2004). The figure was prepared using the DS Visualizer1.7 software (Accelrys).

4.13 Validation of AatJ model by site directed mutagenesis of active site residues

The validity of the homology model was checked experimentally by constructing AatJ mutants with amino acid exchanges in sequence positions predicted to be crucial for ligand binding (i.e. R71, R93, R174, S86, T87, and T88). The far-UV CD spectra of all variants were closely similar to that of wild-type AatJ, suggesting that the mutant proteins folded normally (see Fig. 4.15). Binding of ^{14}C-Glu and ^{14}C-Asp to these Aat variants was then examined in a semi-quantitative fashion by 6% native polyacrylamide gel electrophoresis. Fig. 4.14 shows that the results of these experiments are entirely consistent with the homology model. Only variant AatJ(R174A) retained the ability to bind both Glu and Asp, while AatJ(T87A) still bound Glu, although with lower efficiency. In fact, according to our model, T87 does not directly interact with the ligand, although it connects S86 and T88 which both bind to the α-ammonium group of the amino acid. All other AatJ variants, including several double mutants, did not exhibit significant Glu or Asp binding in the assay employed.

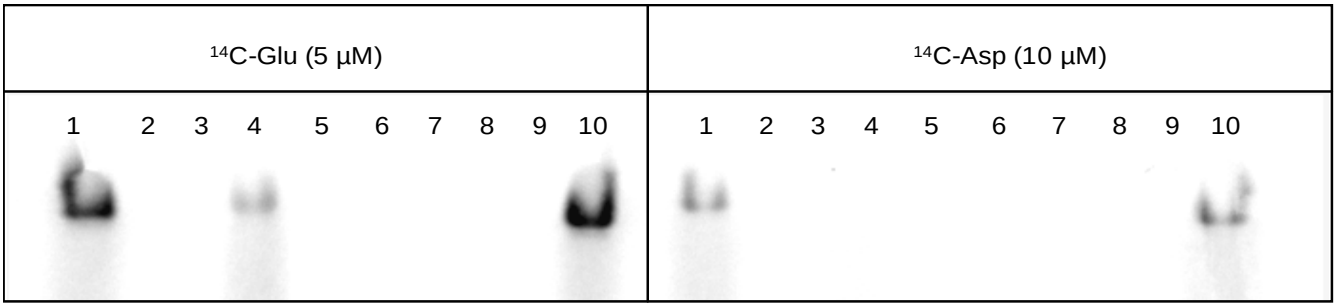


Figure 4.14: Binding of glutamate and aspartate with AatJ and mutants. 5 µM ^{14}C- Glu and 10 µM ^{14}C-Asp was used to analyze binding. Lane 1-10; 1-AatJ(WT), 2-AatJ(R71A), 3-AatJ(S86A), 4-AatJ(T87A), 5-AatJ(T88A), 6-AatJ(R93A), 7-AatJ(S86A,T87A), 8-AatJ(T87A,T88A), 9-AatJ(R71A,R174A) and 10-AatJ(R174A). The figure represents autoradiograph of 5% Native PAGE.

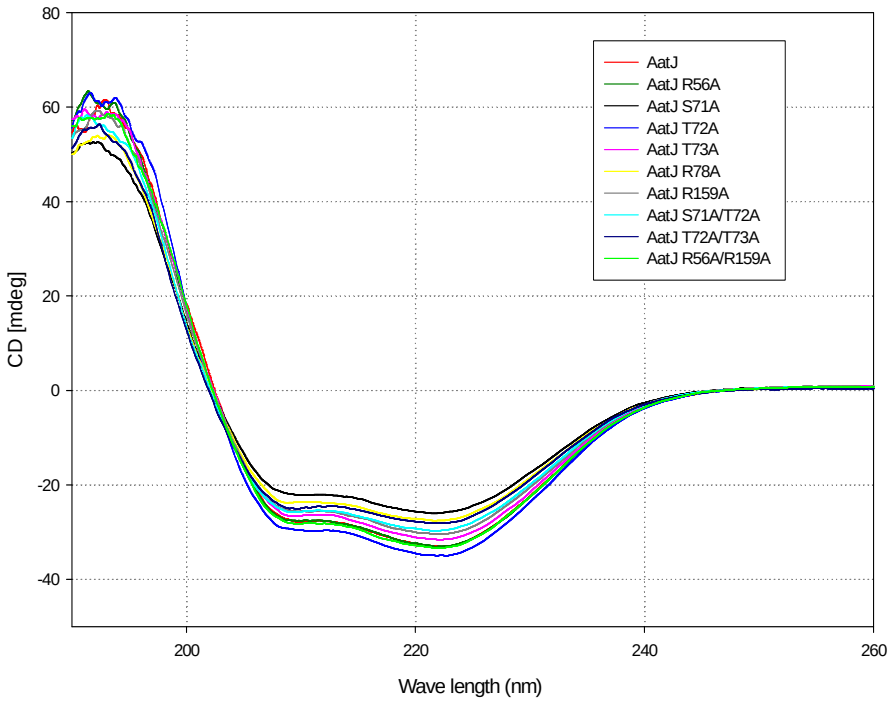


Figure 4.15: Far-UV CD spectra of AatJ (WT) and mutant proteins.

4.14 Expression and purification of the AauR protein

In order to demonstrate direct binding of the response regulator AauR to the promoter region of the *aat* operon, we over-expressed AauR as an N-terminally 6X-His tagged protein, using the plasmid pTrc-His-*aauR.* This vector contains the *lacIq* gene to prevent the unspecific expression of the *tac* promoter. First, the conditions for maximum yield of functional and soluble His-tagged AauR were optimized. The most appropriate conditions for protein production were, induction by 1 mM IPTG, a growth temperature of 37 °C and total expression times not exceeding 6 h. Longer expression times increasingly led to the formation of inclusion bodies. Expression and purification of recombinant AauR was achieved in four steps, i) overexpression of protein in *E. coli* BL21 cells and cell lysis to release His-tagged AauR, ii) purification of the fusion protein by Ni^{2+}-NTA chromatography, iii) digestion with enterokinase to remove the N-terminal tag and, iv) final purification of AauR by gel filtration chromatography on a Superdex-200^{TM} analytical column (GE Healthcare Biosciences). The 4.0 kDa tag introduced by the vector could be selectively removed by enterokinase (Novagen) which cleaves the fusion protein at the sequence D-D-D-D-K↓.

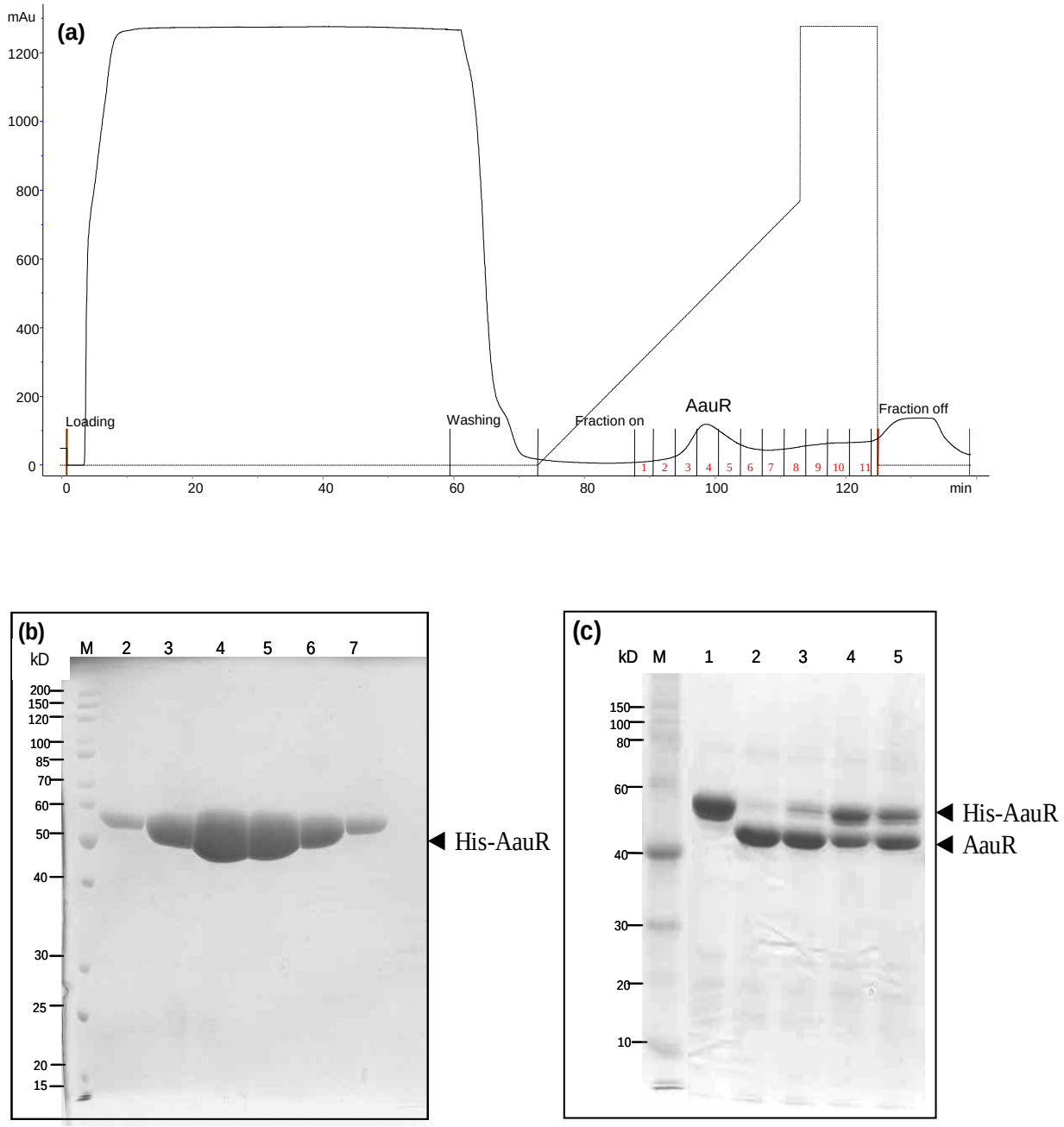


Figure 4.16: Purification of the His-AauR protein. **(a)** A crude protein lysate was loaded on a Ni^{+}-NTA chelating agarose column equilibrated with 50 mM HEPES, pH 7.5 containing 500 mM NaCl and 100 mM imidazole. The column was eluted with linear imidazole gradient. **(b)** The fractions 2-7 from step 'a' analyzed by 15% SDS-PAGE. **(c)** Enterokinase digestion of His-AauR, lane 1; 25 µg undigested His-AauR, lane 2-5; 25 µg His-AauR after incubation with 120 mU, 60 mU, 30 mU and 20 mU enterokinase respectively. Digestion was carried out for 15 h in 20 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$ and 50 mM NaCl at 10 °C.

The liberated peptide was conveniently separated from the protein by the following gel filtration step. As judged by SDS PAGE the resulting preparation was at least 98% pure. The yield of His-AauR after Ni^{+}-NTA affinity chromatography was 5-6 mg/l of culture while the final yield after gel filtration was 3.5 - 4 mg per liter of culture. The purified protein was stored in 50 mM HEPES, pH 7.5, containing 500 mM NaCl and 10% glycerol at -20 °C. The calculated mass of the 6xHis-AauR monomer was 52.7 kDa (Fig. 4.16b) with a pI of 5.6. As AauR is rich in hydrophobic residues, high salt strengths (>0.5 M) were needed for proper solubilization. In its native state the protein is a dimer (105 kD) as confirmed by gel filtration.

4.15 Expression and purification of sensor kinase AauS

The soluble cytoplasmic domain of AauS was expressed using the same vector and the same protocol employed for expression of AauR. In brief, a partial sequence of *aauS* (base pairs 1023 - 1905 relative to the translation start site) was amplified using primers AauS_For and AauS_Rev. The amplified fragment was cloned in pTrcHis-A.

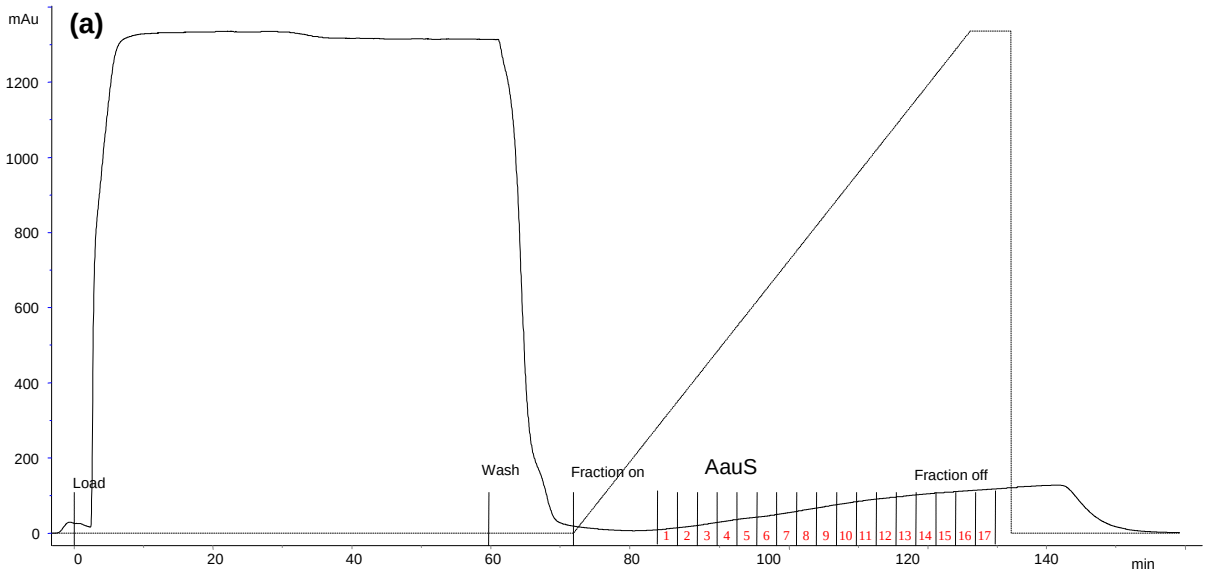


Figure 4.17: (a) Purification of 6xHis-AauS expressed from vector pTrc-*aauS* in *E. coli* BL21. The cell lysate was loaded on Ni^{+}-NTA chelating agarose equilibrated with 50 mM Tris-HCl, pH 7.5 containing 500 mM NaCl and 100 mM imidazole. Bound His-AauS was eluted with linear imidazole gradient.

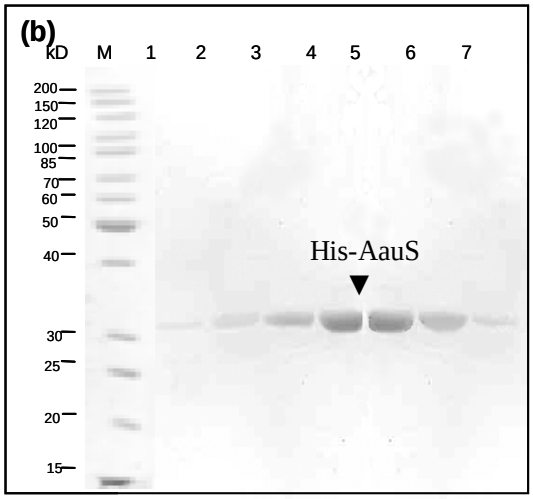


Figure 4.17: (b) Fractions 1-7 from step 'a' analyzed by 15% SDS polyacrylamide gel electrophoresis.

The expression and purification methodology used was similar to that described for His-AauR. The His-tagged AauS was expressed in *E. coli* BL21 cells and purified by Ni^{+}-NTA affinity purification. The fractions containing pure His-AauS were dialyzed against 50 mM HEPES, pH 7.5 containing 500 mM NaCl and concentrated using a Centricon concentrator. The yields of 6xHis-AauS were 2-3 mg/l of bacterial culture. The purity achieved by Ni^{+}-NTA chromatography was >95% as judged by SDS-PAGE (see Fig 4.17b). The purified and concentrated protein was stored at -20 °C after addition of 10% (v/v) glycerol.

4.16 Cross-talk between AauS and AauR

It is well known that sensor kinase and response regulators of two-component systems communicate through phosphoryl transfer. The details of the general phosphorylation mechanism are described in 'introduction'. The *in-vitro* phosphorylation approach is a valuable tool to examine the phosphoryl transfer reactions of two-component systems and to identify the related protein interactions. The Aau two-component system consists of the sensor kinase AauS and its cognate response regulator (AauR) as shown in Fig. 4.18. The ability of AauS to auto-phosphorylate and the phosphoryl transfer to AauR were studied with purified proteins.

Figure 4.18 shows the structure of AauS and AauR as predicted from sequence alignments with other well-known systems while Fig. 4.19 shows partial alignments of functionally important

regions (“boxes”). They include the conserved histidine and aspartate residues which are involved in phosphorylation and phosphate transfer.

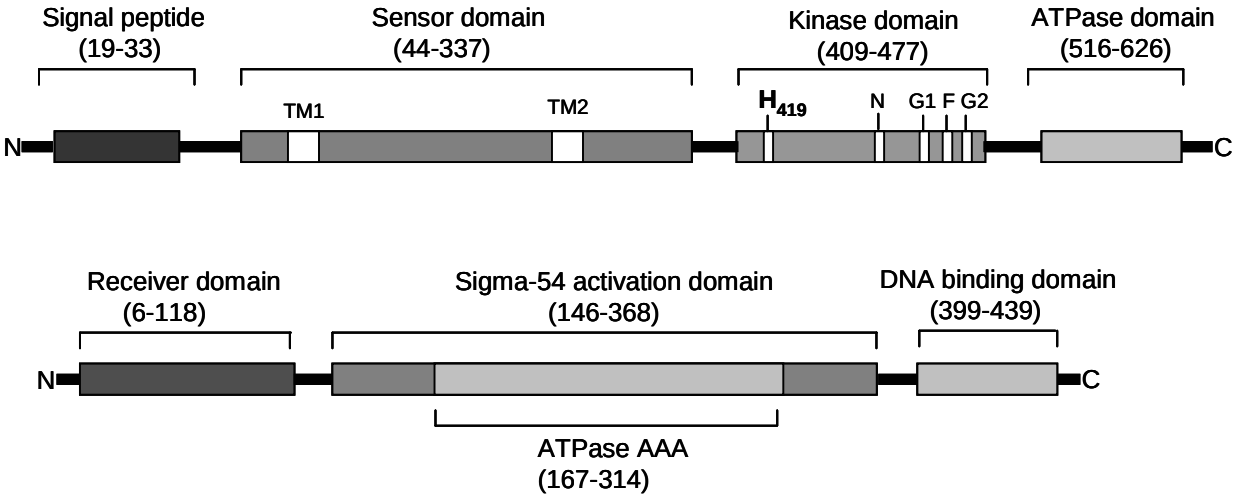


Figure 4.18: Predicted domain structure of AauS and AauR based on sequence similarity with other available structures.

(a)

```
                  H-box              N-box          G1-box          F-box          G2-box
                    ▼                  ▼              ▼               ▼              ▼
DCTB_RHILE VAAGVAHEINQPV (*H,415) VLINLLQNALEA..NGPGIPTEIRKGLFTPFNTSK..ESGLGLGLVI
HisK_ECOL6 MATEIAHEQNQPL (*H,394) IFSNLISNALDS..SGGGFAPEVVDRIFEPFFTTK..RRGMGLGLAI
AauS_PSEPK MSTSIAHELNQPL (*H,419) ILVNLIGNALDA..NGHGIDPEARKHLFEPFFTTK..EHGLGLGLTL
```

(b)

```
                                  D-box
                                    ▼
CHEY_ECOL6  MADKELK---FLVVDDF...ISDWNMPNMDGLE...GYVVKPFT (D12, D13, *D57, K109)
CHEY_SALTY  MADKELK---FLVVDDF...ISDWNMPNMDGLE...GYVVKPFT (D12, D13, *D57, K109)
AauR_PSEPK  MNQAPLT---VLIVEDD...VSDIRLPGIDGLE...DFMEKPFS (E12, D13, *D56, K106)
```

Figure 4.19: Partial sequence alignments of *P. putida* KT2440 AauS and AauR with related proteins. **(a)** AauS_PSEPK; AauS of *P. putida*, DCTB_RHILE; DctB of *R. leguminosarum*, HisK_ECOL6; histidine kinase from *E. coli*. **(b)** AauR_PSEPK; AauR of *P. putida*, CHEY_ECOL6; CheY of *E. coli* and CHEY_SALTY; CheY of *S. typhimurium.* Conserved histidine and aspartate residues (sites of phosphorylation) are marked with asterisks (*).

4.16.1 *In-vitro* autophosphorylation of the AauS cytoplasmic domain

In bacterial two-component systems, the activated sensor kinases are autophosphorylated at a histidine residue in the kinase domain or in a separate Hpt domain (see 'Introduction' for details). Alignments with other known and functionally characterized sensor kinases suggest that the site of phosphorylation in AauS is His_{419} (Fig. 4.19a).

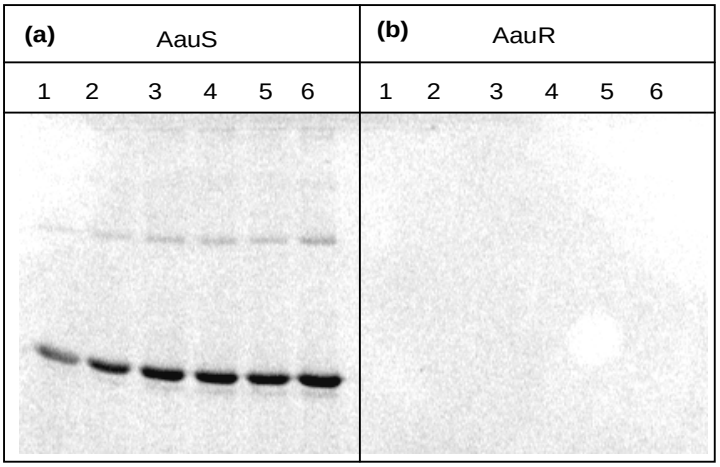


Figure 4.20: Phosphorylation of AauS and AauR (see 'Methods'). **(a)** Lane 1; phosphorylation after 0 min, lane 2; after 5 min, lane 3; after 10 min, lane 4; after 15 min, lane 5; after 20 min and lane 6; after 30 minutes. Samples were quenched with 3X SDS-EDTA buffer, separated by SDS-PAGE and autoradiographed. **(b)** Lane 1-6 represent AauR, treated like AauS for detection of a possible auto-phosphorylation activity.

The experiment illustrated by Fig. 4.20a shows that the purified AauS soluble domain autophosphorylates *in-vitro*. At 37 °C, maximum levels of phosphorylation were reached after a 15 to 20 min of incubation. The phosphorylated AauS domain was stable at room temperature for several hours. As expected, the response regulator AauR was unable to phosphorylate itself when incubated with γP^{33}-ATP under the same conditions (Fig. 4.20b).

4.16.2 *In vitro* phosphoryl transfer from AauS to AauR

As illustrated by Fig. 4.21, phosphorylated AauS transfers a phosphate group to AauR with high efficiency. The intensities of the bands in Fig. 4.21 indicate that the phosphoryl transfer reaction between AauS and AauR involves an 1:1 molecular interaction between both proteins.

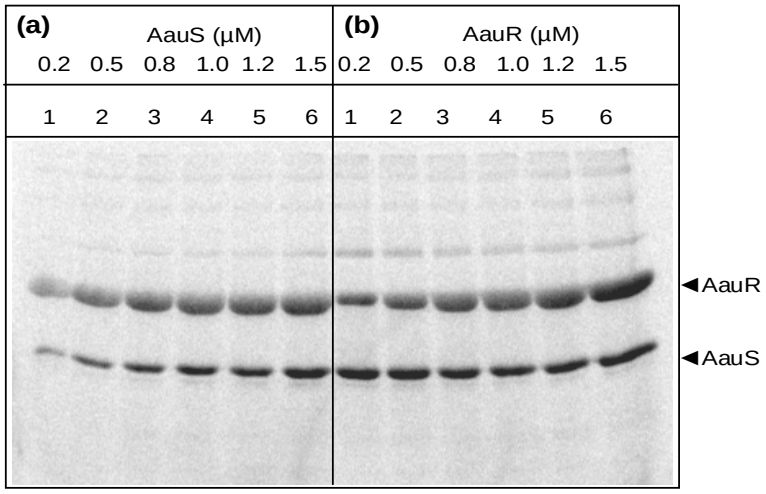


Figure 4.21: (a) Lanes 1-6; 1μM AauR incubated with 0.2-1.5 μM of phosphorylated AauS. **(b)** Lanes 1-6; 1μM phosphorylated AauS incubated with 0.2-1.5 μM AauR. The AauS protein was phosphorylated as described in 'Methods' and added to AauR in TGMNKD buffer. The phosphoryl transfer reaction was terminated after 10 sec by addition of 3X SDS-EDTA buffer, then the samples were separated by SDS-PAGE and autoradiographed.

4.16.3 Stability of phospho-AauR

The phosphorylated AauS successfully transferred the phosphate group to AauR, yielding a product that was stable for 5 - 10 min (Fig. 4.22). The phosphoryl transfer was complete in less than 1 min and after 5 min the signals rapidly decreased, probably due the dephosphorylation of AauR.

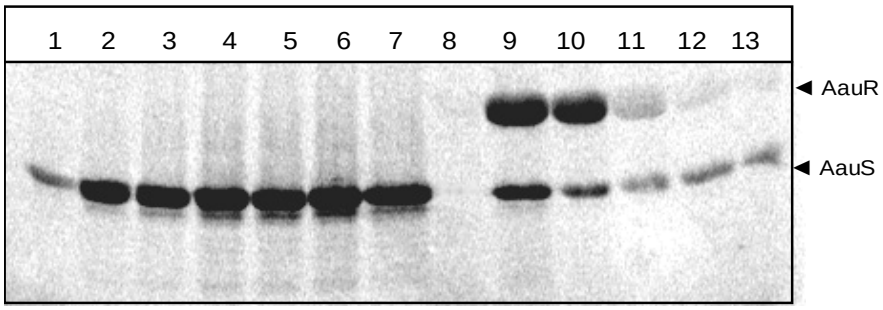


Figure 4.22: Phosphorylation of sensor kinase AauS and phosphoryl transfer to AauR. Lane 1; phosphorylation of AauS at time 0, lane 2; after 5 min, lane 3; 10 min, lane 4; 30 min, lane 5; 40 min, lane 6; 60 min and lane 7; phosphorylation after 80 min. Lane 8; AauR incubated with [γ-P^{33}]-ATP for 60 min in phosphorylation buffer. Lane 9; AauR (1μM) incubated with 1 μM phosphorylated AauS at time 0, lane 10; phosphoryl transfer after 1 min, lane 11; phospho-AauR stability after 5 min, lane 12-13; phospho-AauR stability after 10 min and 30 min.

4.17 Binding of AauR to the promoters of *aatPMQJ* and *ansB*

Promoter prediction tools suggest that *P. putida* KT2440 genes PP1068-PP1071 are expressed as a single operon. As expected, the promoter region of PP1071 (*aatJ*) contains the σ^{54} binding site T**GG**CACgactcA**TG**CC (recognition bases shown in bold) located at -12/-24 relative to the transcription start site. For expression of σ^{54}-dependent genes or operons another activated transcription factor is required to change the closed promoter complex into an open one (see 'Introduction' for details).

As shown Fig. 4.23a, the purified AauR protein can indeed bind to the promoter upstream region of *aatJ* as shown by the gel mobility shift assay. The binding is sequence- specific as AauR does not interact with nonspecific DNA fragments Fig. 4.23a. AauR also appeared to interact with the promoter region of *ansB* in a concentration dependent fashion (Fig. 4.23b) although the shift was smaller than observed with the *aat* promoter.

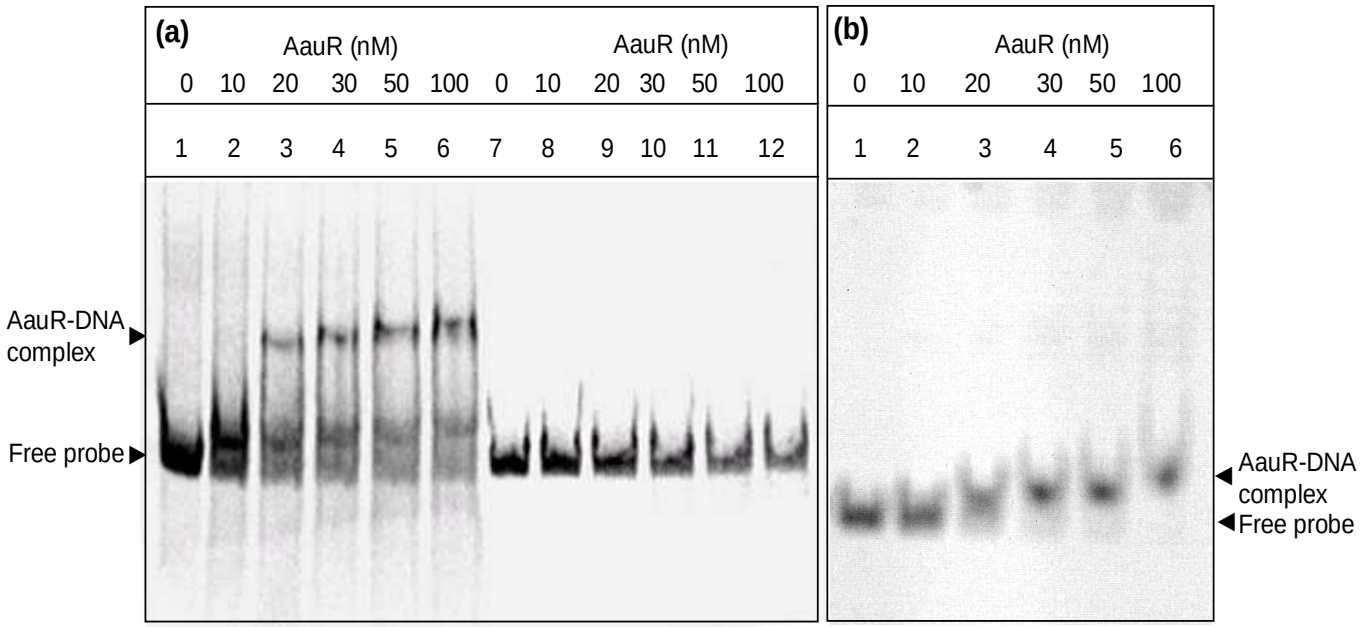


Figure 4.23: Binding of AauR to the promoter upstream region of *aat* (-84 to -343 bp relative to the translation start site). **(a)** Lane 1; DNA probe w/o AauR (5.0 fmol), 1anes 2-6; DNA probe + AauR (20 to 100 nmol), lane 7; unspecific DNA fragment (5.0 fmol), lanes (8-12); unspecific DNA fragment + AauR (20-100 nmol). **(b)** Binding of AauR to the *ansB* promoter (-25 to -208 bp relative to the translation start site), lane 1; DNA probe, lanes 2-8; probe + 20-100 nmol AauR.

4.18 Characterization of the AauR binding motif in the *aat* promoter

The binding region and exact binding motif of the response regulator AauR was characterized by two different approaches. First, sequential deletions of the putative promoter region gave an idea of the approximate location of the binding site. Then, a DNase I protection assay revealed the exact position of the DNA motif interacting with AauR.

4.18.1 Sequential deletion analysis of the *aatJMQP* promoter region

The transcription and translation start sites in the promoter region of the *aatJ* operon were predicted using an online promoter prediction tool (BPROM) available for prediction of bacterial promoters in the intergenic regions (http://www.softberry.com). To identify the location of the AauR binding motif, parts of the region from -83 to -202 relative to the predicted transcription start were deleted in a stepwise fashion.

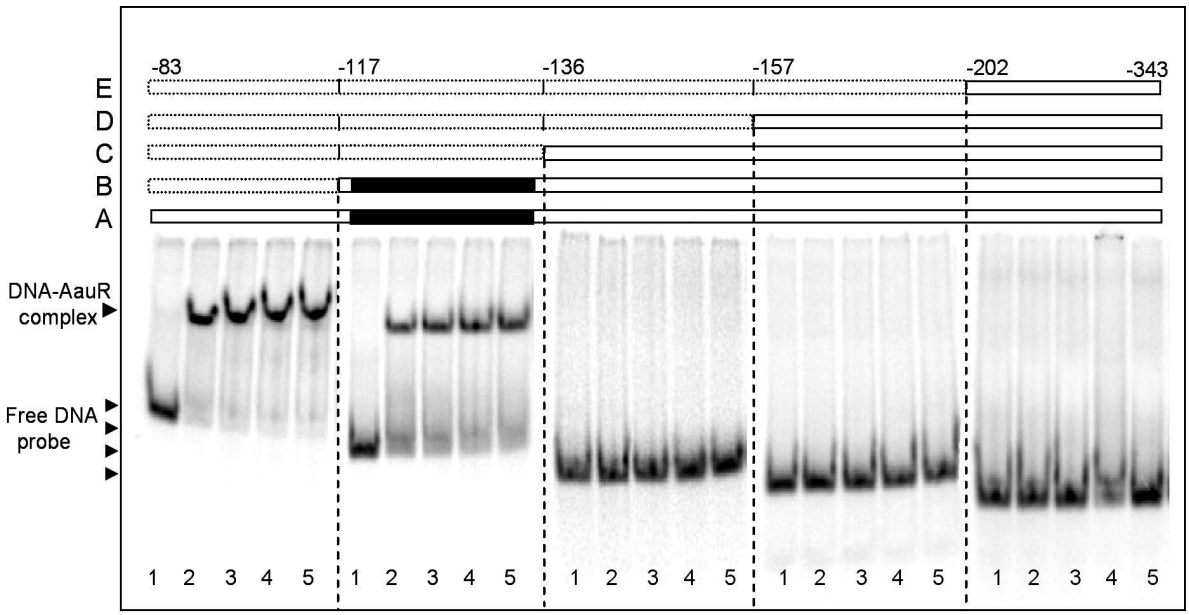


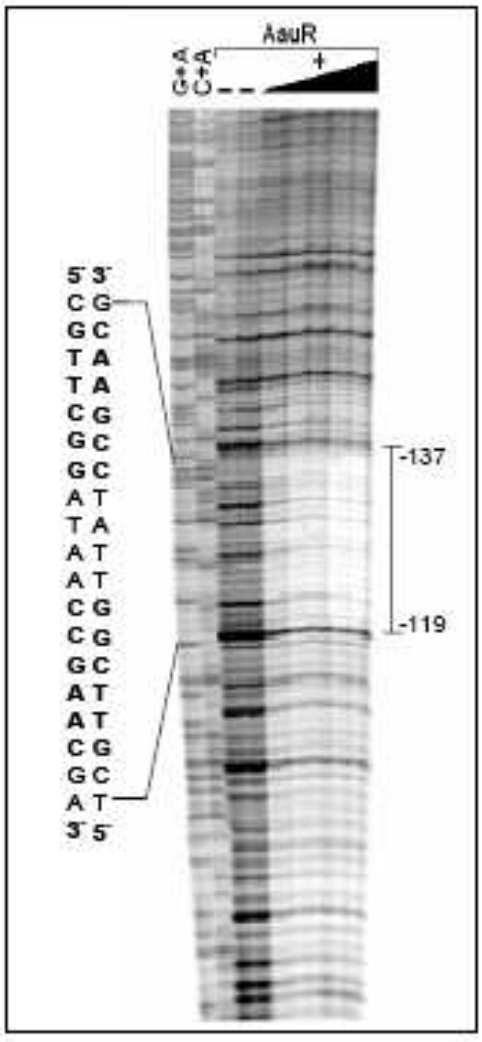
Figure 4.24: Gel mobility shift assay of AauR binding to *aat* promoter fragments. Lengths and positions (relative to the transcription start site) of fragments A – E are depicted schematically on top. Within each block, the leftmost lane contained 5 fM of ^{33}P-labeled DNA w/o AauR while lanes 2-5 contained labeled probe DNA + increasing amounts of AauR (2-10 nM, 3-20 nM, 4- 30 nM, and 5- 50 nM) A: -202 to -343, B: -157 to -343, C: -136 to -343, D: -117 to -343, and E: -83 to -343. Mobility was analyzed by 6% native polyacrylamide gel electrophoresis and autoradiography.

Different fragments of the *aat* promoter (increasingly shortened from the -83 region) were amplified from genomic DNA of *P. putida* KT2440 using primers PromFor_PP1071 as forward primer and PromRev_PP1071, PromRev_117, PromRev_136, PromRev_157 and PromRev_202 as reverse primers Table 2.3.3 (see 'Methods'). The purified fragments were labeled by ^{33}P and analyzed for AauR binding by gel mobility shift assays. Again, the data suggested that the binding of AauR to the *aat* operon is highly specific as an unspecific DNA fragment of about the same length did interact with AauR. As shown by Fig. 4.24 only fragments containing the region between -117 and -136 were shifted by AauR indicating that this partial sequence contains the AauR binding site.

4.18.2 DNase I footprinting

Identification of the exact binding motif for AauR in the *aat* promoter was performed by a DNase I protection assay in the -83 to -343 region. As shown in Fig. 4.25a, AauR selectively and efficiently protected base pairs -119 to -137 from DNase I digestion. Obviously, this finding is consistent with the sequential deletion data shown in Fig 4.24. The protected sequence CGTTCGGATAACCGAACGA is situated at (-119 to -137) relative to the transcription start site, Fig. 4.25b. As expected for the binding site of a dimeric σ^{54}-dependent response regulator the protected region contains an 2x6 nt inverted repeat with a spacing of 4 nucleotides (i. e. **GTTCGG**NNNN**CCGAAC).** This suggests that both parts of the binding site lie on the same side of the DNA double helix and can interact with the helix-turn-helix motif of AauR.

(a)



(b)

```
atctgtggaactctccaggcaaagagcaaagctcactacgtctgagcccttgccgcgcactgcatagtta
                                -137 ▼                    ▼ -119
ccgcaagcgataccgggataggttggcagcccatgcGTTCGGataaCCGAACgagcaatcgctgtttatt

cggcctggcgaaccccggagagaggtcgagactgtcaagcacgagcaataccagtggtttaaggtcgaca
          -24         -12          ↱ Transcription start
gcagggcttGGcacgagtcaTGctctacacttGgtagccgaatgcacccgcttcttgctgtattcgttga
                                                    ↱ Translation start
acgaagagtccgccaacggctccataaaaaaaacaaacacgtcgaggaaaatTTG
```

Figure 4.25: (a) DNase I protection region by AauR in *aatJ* promoter upstream region. Lane 1; ladder (G+A), lane 2; ladder (C+A), lane 3 & 4; promoter sequence of *aatJ* (-83 to -343) 50 ng labeled with ^{33}P, lane 5-9; promoter DNA with 20-100 nM AauR. Samples of lane (3-9) were digested with 0.05U DNase I for 2 minutes at 37 ^{o}C and separated on an 8% urea- polyacrylamide sequencing gel. **(b)** *aat* promoter DNA sequence showing the σ^{54} binding site at -12/-24, the transcription, translation start sites, and the protected region at -119 to -137 (black background).

4.19 Alignment of *aatJQMP* and *ansB* operons of *Pseudomonas spp.*

We then searched for the presence of this consensus sequence in intergenic regions of other *Pseudomonas* species. The intergenic regions of the *aat* and *ansB* promoters were aligned by using the online tool NOMAD (http://www.expasy.org/tools/nomad.html). As shown by Fig. 4.26, the AauR binding motif is indeed conserved in the *ansB* and *aatJ* promoters of other pseudomonads. These promoters also contained a σ^{54} binding site at -12/-24 (data not shown here).

(a)

PP1071	GCGATTGCTC**GTTCGGTTATCCGAAC**GCATGGGCTG	(-120 to -136)
PFL4871	GCCCATGCGC**GTTCGGTTTTCCGAAT**GGCGAAGCCG	(-179 to -195)
PA1342	GGGGCATGGC**GTTCGGTTTTCCGAAC**GTTTCGCCGA	(-142 to -158)
PSPTO4171	ATTCGACTGC**ATTCGGCTTTCCGAAC**GCGTCTCCCT	(-134 to -150)
PSYR3908	AACCCCTTCC**ATTCGGTTTTCCGAAC**AATCGACCCC	(-131 to -147)
PSPPH3902	ACTCCGTTGC**ATTCGGTTTTCCGAAC**ATTTCGCTCT	(-81 to -97)
PSEEN1193	GCCATATGGC**GTTCGGAAATCCGAAT**GAGTGATGGG	(-80 to -96)

(b)

PP2453	AGGAAAAGGT**GTTCGGTTTTTCGGAA**AAGACTGCAT	(-151 to -167)*site 1*
PP2453	GCGGCATTGC**ATTCGTAATTCCGAAC**AAATAAAGGA	(-119 to -135)*site 2*
PA1337	GGGGCATGGC**GTTCGGTTTTCCGAAC**GTTTCGCCGA	(-142 to -158)
PSEEN1951	GATGGCGTTC**GTTCGGCTTTCCGAAC**ATTTGCAGGA	(-130 to -146)*site 1*
PSEEN1951	AGGAAAAGGT**GTTCGGTTTTTCGGAT**AAATTGAGGA	(-98 to -114)*site 2*
PFL2099	ACGGTGGCGC**GTTCGGCAATCCGAAA**ATGCTCGTCA	(-196 to -212)

Consensus .....***g*TTCGGNNNNCCGAA*c***.....

Figure 4.26: Alignment of promoter regions of **(a)** *aatJQMP* promoter upstream sequence of different *Pseudomonas spp.* PP1071; *P. putida* KT2440, PFL4871; *P. fluorescens* Pf-5, PA1342; *P. aeruginosa* PA01, PSPTO4171; *P. syringae* DC3000, PSYR3908; *P. syringae* pv B728a, PSPPH3902; *P. syringae* pv *phaseolicola*, PSEEN1193; *P. entomophila* L48. **(b)** Asparaginase II (*ansB*) promoters of PP2453; *P. putida* KT2440, PA1337; *P. aeruginosa* PA01, PSEEN 1951; *P entomophila* L48 and PFL2099; *P. fluorescens* Pf-5. In *Pseudomonas syringae spp.* periplasmic glutaminase/asparaginase is absent. The position of AauR binding site was determined on the basis of promoter prediction and the presence of σ^{54} binding region at -12/-24.

In *P. putida* and *P. entomophila*, the AauR binding motif within *ansB* occurs twice (Fig. 4.26b), whereas periplasmic glutaminase/asparaginase II is not encoded by the genome of *P. syringae*.

5. Discussion

Pseudomonas putida can utilize a great variety of natural compounds to meet its energy requirements and to synthesize new molecules. The natural occurrence of *P. putida* in the rhizosphere is convincing evidence of the capacity of this organism to cope with many natural perturbations. It quickly adapts to changing biotic and abiotic conditions in the soil such as nutrient availability by modulating the expression of genes which ultimately help to cope with these changes. The very first step in the recognition of such changes is the sensing of appropriate signals by receptor-effector proteins known as two-component systems. These systems may control just a single gene, operons or sometimes large sets of genes, so called regulons that regulate global processes. In two-component systems the membrane-bound sensor first recognizes soil stress signal and transmits it to the cytoplasmic regulator protein, which ultimately facilitates or inhibits the expression of genes. The whole process from recognition to the ultimate response is very quick which helps bacterial cells to minimize the exposure to unfavorable conditions. The *P. putida* KT2440 genome encodes more than 130 sensor kinases and response regulators that make up at least 50 different two-component systems. At present, most of these putative regulatory systems have not been associated with any defined function.

In this study, we show that a two-component system encoded by PP1067-PP1066 (*aauS-aauR*) is required for efficient utilization of *P. putida* KT2440 on acidic amino acids (Glu and Asp) and their amides (Gln and Asn). As far as this strain is concerned, AauS-AauR is the first two-component system shown to participate in acidic amino acid utilization. According to our present results, the system has at least two targets: it controls the expression of PGA, and thus the hydrolysis of exogenous Asn and Gln in the periplasmic space. In addition, the *aau* system directly or indirectly affects the activities of key enzymes of acidic amino acid metabolism, such as aspartase and glutamate dehydrogenase. It also regulates the expression of an ABC transporter, AatJMQP, which is highly specific for Glu and Asp import. The Aau two- component system and the Aat transporter appear to occur in all pseudomonads and many other bacteria where they probably fulfill the same role as in *P. putida*. Detailed studies of glutamate and aspartate binding suggested that Aat is a high-affinity transporter for acidic amino acids. The active site (binding pocket) of the Aat solute-recepor protein is different from those other amino acid ABC transporters

with conserved Arg as key residue. In this work we further identified the molecular basis of transcriptional control by the AauS-AauR system.

5.1 Acidic amino acids as sources of carbon and nitrogen for *P. putida*

In enteric bacteria, most of the nitrogen required by the cellular synthesis machinery is usually provided by the ammonia assimilation pathway which yields mainly glutamate and some glutamine (Magasanik 1993). The inter-conversions between glutamate and glutamine via the GS/GOGAT and GDH pathways are further crucial steps in nitrogen assimilation (Merrick & Edwards 1995 and Reitzer 2003). Growth of *E. coli* on glutamate as sole carbon source only occurs with strains carrying mutations that increase the expression of genes encoding glutamate transport systems (Kahane *et al.* 1976 and Booth *et al.* 1989). *E. coli* is also unable to utilize glutamine in normal physiological conditions, as its Gln uptake system is shock-sensitive and conditional (Masters & Hong 1981). Under conditions of nitrogen starvation, many bacteria switch to a more efficient utilization of amino acids (Booth *et al.* 1989). This so-called nitrogen response has been studied in detail with *E. coli* (see 'Introduction' for details).

Unlike *E. coli*, pseudomonads efficiently utilize Glu, Gln and other amino acids as sole sources of carbon and nitrogen or in combination with alternate carbon source such as glucose or succinate. Growth of *P. putida* on acidic amino acids is comparable to or better than growth on ammonium as nitrogen source and glucose as carbon source (cf. section 4.1). This finding is in agreement with previous work conducted in our laboratory (Klöppner, 1999). *P. putida* also utilizes most dicarboxylic acids occuring in the tricarboxylic acid cycle (succinate, fumarate, α-ketoglutarate) as carbon sources with growth rates comparable to those on glucose, whereas gluconeogenic dicarboxylates are poor carbon sources for *E. coli*.

5.2 The Aau two-component system regulates Glu/Asp assimilation in P. *putida*

In this work, the role of the *P. putida* AauS/R two-component system in the control of acidic amino acid transport and utilization was studied in detail. Growth of AauS/AauR-deficient mutants on acidic amino acids was greatly impaired while growth in other amino acids or on NH_4^+/glucose was not affected by these mutations (cf. sections 4.1 and 4.2). Supply of additional carbon sources (glucose or succinate) partially restored growth of the mutants, while NH_4^+ had no significant

effect. This indicates that AauS/R system is distinct from the regulators involved in the ammonia assimilation system.

We further observed that AauR$^-$ mutants were defective in acidic amino acid uptake (Fig. 4.3), which is probably the main reason for the growth defect. Sonawane, showed that in *P. putida* acidic amino acids and their amides induce the expression of certain genes among them PP1071, the solute receptor of an ABC transporter encoded by an operon adjacent to *aau* (Sonawane 2003b). This ABC transporter was named as *aat* ABC (for acidic amino acid transport) by us. Indeed, *Aat* deletion mutants (KT*aatJ* and Kt*aatP*) also exhibited defects in Glu and Asp uptake (Fig. 4.4). The importance of the *aau* system is further emphasized by our observation that KT*aauR* mutants were greatly impaired in their ability to survive under conditions of starvation (cf. Fig. 4.9). Similar results were reported for *gltB* mutants of *P. putida* (Sonawane & Rohm 2004 and Eberl *et al.* 2000). On the other hand, the *aauR*- phenotype was complemented significantly when an intact functional copy of the *aauR* gene was introduced in mutants (Sonawane *et al.* 2006). Thus the AauR/S two-component system appears to function as a key regulator in acidic amino acid utilization.

5.3 AauR$^-$ mutants exhibit multiple changes in amino acid metabolism

The physiological significance of the AauR/S system in *P. putida* is also confirmed by our finding, that the activities of several key enzymes of acidic amino acid metabolism are markedly changed in KT*aauR* mutants (see 'Results'). The glutaminase/asparaginase (PGA) activity is completely lost in such mutants (Fig. 4.5b). PGA is a highly active periplasmic protein that converts glutamine and asparagine into the respective dicarboxylates (glutamate and aspartate). In *Pseudomonas fluorescens* ATCC13525 and *P. putida* ATCC12633, PGA enzyme is strongly induced by Gln and Asn but repressed by glucose and dicarboylates like succinate a result of carbon catabolite repression (Hüser *et al.* 1999 and Sonawane *et al.* 2003a). *P. putida* mutants with inactivated PGA failed to grow in acidic amino acids indicating an essential role of PGA in the utilization of these amino acids. This enzyme may also be important in host-plant interaction as good root colonizers showed higher PGA activities (Klöppner, 1999). The significant role of this enzyme is to convert the amides into their amino acids for their fast uptake.

An especially interesting feature of KT*aauR* mutants was the intracellular accumulation of glutamate in late log phase when grown in Asp or Asn as sole source of carbon and nitrogen (Fig. 4.8). The elevated glutamate level is probably due to higher activities of GDH as shown in Figs. 4.6 and 4.7. A study by Bellion & Tan (Bellion & Tan 1984) showed that GDH activity was elevated in the presence of ammonium chloride as nitrogen source in *Pseudomonas sp*. AM1 and repressed when methyamine or nitrate were used as nitrogen sources. A recent study by Syn *et al.*, (Syn *et al.* 2004) showed that in *P. putida* PNL-MK25; GDH activity is dependent on various carbon sources and the expression of GDH increased 5-26 folds under conditions of nutrient limitation, which might also be the case in mutant KT*aauR*. In fact, the GDH pathway should be the preferred one under energy-limited conditions because GDH requires 20% less ATP than the GS-GOGAT pathway.

Together with asparaginase, aspartase play a major role in maintaining the aspartate and asparagine pools inside the cell (Sun & Setlow 1991). The activity of aspartase in *E. coli* increases several fold when the cells are grown on aspartate as sole carbon source, while addition of glucose as carbon source decreased aspartase activities (Nishimura & Kisumi 1984). A similar repressive effect of glucose on aspartase activity was seen with strain KT*aauR* grown in Asp as sole source of carbon and nitrogen (cf. fig 4.7). Aspartase converts aspartate to fumarate and feeds it into TCA, while GDH favours the synthesis of glutamate from 2-oxoglutarate at equilibrium (Merrick & Edwards 1995). As a consequence of these two enzyme activities, the overall level of Glu rises. Natera and co-workers (Natera *et al.* 2006) showed that *Bradyrhizobium spp*. can accumulates glutamate when grown under acidic stress conditions. Our explanation of elevated GDH role in glutamate accumulation is compatible with their findings. Accumulation of Glu was observed mainly with organisms placed under stress conditions (commonly alterations of pH) where glutamate functions as osmo-protectant (Csonka *et al.* 1994 and McLaggan *et al.* 1994), However, such kind of stress does not seem to be operated in *P. putida* which is normally growing in M9 medium (unchanged pH). In *R. leguminosarum*, the highest intracellular Glu accumulation was observed with aspartate as the nitrogen source (Reid *et al.* 1996). Studies by Poole an coworkers showed that mutants of *Rhizobium leguminosarum* with defects in tricarboxylic acid (TCA) cycle enzymes exhibited greatly elevated intracellular glutamate levels and also excreted glutamate (Walshaw *et al.* 1997a and Hosie *et al.* 2001). In *Rhizobium spp.* Glutamate excretion is thought to

constitute an overflow pathway with the function of removing excess carbon and reducing equivalents. Our finding that, in *P. putida*, significant excretion of glutamate occurred only in the presence of glucose (Fig. 4.3) may be rationalized by the fact that glucose degradation replenishes the TCA cycle to create additional 2-oxoglutarate. Another evidence for the existence of an overflow pathway in *P. putida* KT2440 *aau* mutants is provided by the observation that KT*aauR* growing on Glu (but not on NH_4^+/glucose) strongly expresses solute-binding proteins associated with amino acid ABC transporters. Two of these (BraDEFG, encoded by PP1140 to PP1137, and a transporter encoded by PP4866 to PP4863) are thought to transport branched-chain amino acids, while the third (AapQMP, encoded by PP1298 to PP1300) is annotated as a general amino acid transporter. As shown by (Walshaw & Poole 1996), it is transporters of the Aap and Bra types that mediate the selective efflux of glutamate in *R. leguminosarum*.

5.4 The AauR-AauS system is ubiquitous in all pseudomonads

The genomes of *Pseudomonas spp.* are among the largest bacterial genome sequenced; they show a sequence similarity of more than 85%. Most pseudomonads are non-pathogenic soil inhabitants (see 'Introduction') while *P. aeruginosa* and *P. syringae* are pathogens. Several two-component systems of *Pseudomonas spp.* have been identified in last 15 years and correlated with host-parasite interactions, virulence and cellular integrity (Kivistik *et al.* 2006, Horak *et al.* 2004, Goodman *et al.* 2004 and Laskowski *et al.* 2004). Some two-component systems (such as Ntr and Dct) are global regulators of nitrogen or carbon metabolism while others regulate particular phenomena inside the cells. Both Ntr and the Dct system depend on *rpoN* (the σ^{54} transcription factor). Growth and adaptability of pseudomonads are also determined by σ^{54} which assists in the expression of a great variety of genes involved in the uptake and utilization of carbon and nitrogen. As judged from genome scans, *P. putida* KT2440 contains at least 72 genes with potential σ^{54} binding sites (http://www.promscan.uklinux.net/RpoN/promscan.outfile.66.html).

AauR, the response regulator of the *aau* system also contains a σ^{54} interaction domain (Fig. 4.18), indicating that it regulates σ^{54}-dependent operons by direct interaction. However, indirect control (pleiotropic effects) by AauR-AauS cannot be ruled out. All available genomes of *Pseudomonas spp.* encode a single copy of *aau*. The alignment of AauS and AauR of different

pseudomonads is shown in Fig. 5.2, the sequence similarity between the respective sensor kinases and response regulators is more than 85-90%, with functional domains being highly conserved.

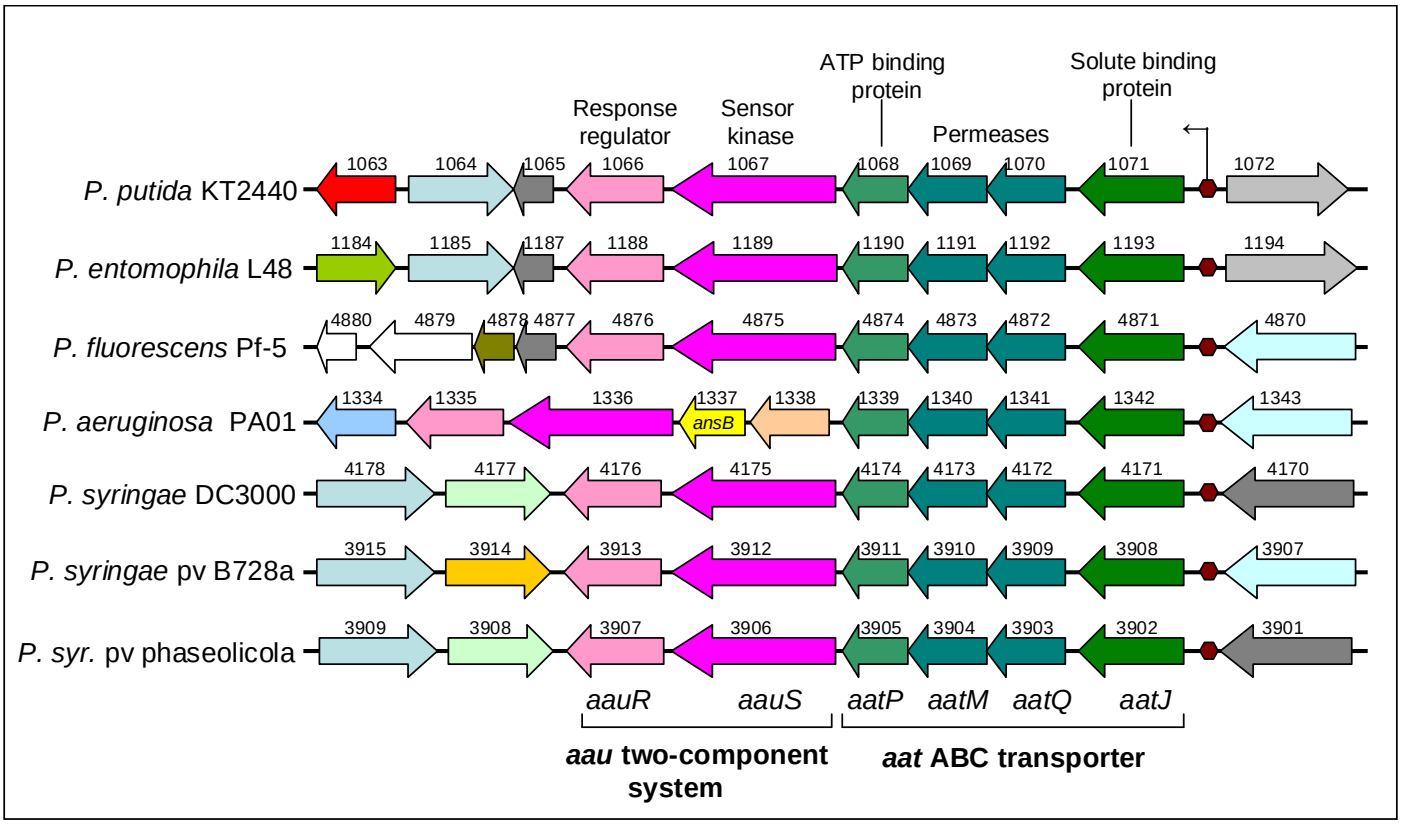


Figure 5.1 Genetic organizations of AauR/S two-component system and AatJMQP ABC transporter in *Pseudomonas spp.* The site of transcriptional control by AauR is shown upstream of the *aat* promoter. In *P. aeruginosa* the PGA-encoding *ansB* gene is inserted between *aat* and *aau*.

5.5 AauR-AauS is a typical two-component system

A*au* is a simple two-component system with AauS as the sensor kinase and AauR as the response regulator as described in other organisms by (Stock *et al.* 2000, Foussard *et al.* 2001 and West & Stock 2001). In the present work, the cross-talk between AauS and AauR was analyzed by *in-vitro* phosphorylation and transphosphorylation reactions. A truncated His-tagged AauS (excluding transmembrane and signal domain) was expressed, purified and phosphorylated by using [γ-P^{33}]-ATP. The degree of phosphorylation reached a maximum after 30 min, and the phosphorylated AauS was stable up to 4 h at room temperature. As expected, the response regulator was not phosphorylated in the same conditions. The phosphorylated AauS successfully transferred the

phosphate group to AauR yielding a product that was stable for 5 - 10 min (cf. Fig. 4.22). Phosphorylated AauR is probably dephosphorylated by a phosphatase activity in the sensor kinase, as AauS showed *in-vitro* phosphatase activity (data not shown). Similar results were reported by Eguchi et al, (Eguchi *et al.* 2004) with the PhoQ sensor kinase of *Salmonella typhimurium* which exhibits phosphoryl transferase as well as phosphatase activity to modulate the function of PhoP response regulator. Another study by Hsing and Silhavy (Hsing & Silhavy 1997) showed the same kind of regulation in the OmpR-EnvZ two-component system of *E. coli*.

In some cases it was observed that the presence of the target DNA increases the stability of a phosphorylated response regulator. In the *E. coli* OmpR-EnvZ system, the presence of specific binding DNA during transphosphorylation increased the stability of phosphorylated OmpR (Mattison & Kenney 2002 and Zhu *et al.* 2000). On the other hand, phosphorylation can increase the DNA binding efficiency of a response regulator several fold in comparison to the unphosphorylated form as described by Dahl *et al*, (Dahl *et al.* 1997) with UhpB/UhpA and Emmerich *et al.* (Emmerich *et al.* 1999) for the RegSR two-component system of *Bradyrhizobium japonicum*. As found in these *in-vitro* studies, AauS also seems to mediate the function of AauR by both phosphorylation and dephosphorylation. In this work, however, we did not notice a difference in binding efficiency of phosphorylated and unphosphorylated AauR (data not shown). Likewise, addition of ligands or possible interacting molecules (Glu, Gln, Asp and Asn) did not significantly affect the phosphoryl transfer (AauS→AauR) or the stability of phosphorylated AauR.

5.6 AauR-AauS is different from the DctB-DctD two-component system

The fact that, succinate is still efficiently utilized by KT*aauR* and KT*aauS* indicates that *aau* is functionally distinct from the *dctBD* system, which regulates dicarboxylate uptake and metabolism in various bacteria. In rhizobia, for instance, mutations in any of the *dct* genes result in a loss of the ability to transport and utilize C_4-dicarboxylates (Reid *et al.* 1996 and Reid & Poole 1998).

Fig. 5.2, shows partial sequence alignments of AauS (encoded by PP1067) and AauR (PP1066) with orthologues from other pseudomonads and two closely related paralogues in *P. putida,* encoded by PP0263-PP0264 and PP1401-PP1402. Although the function of the latter systems is unknown, they also may have role in dicarboxylate regulation. From Fig 5.2, it is clear that the Aau systems of different *Pseudomonas spp.* are closely related while the sequence

similarity with other putative dicarboxylates regulators in *P. putida* is less pronounced. This is especially true for the DNA-binding helix-turn-helix (HTH) motifs of the response regulators indicating that the binding sites in the target promoters are also different.

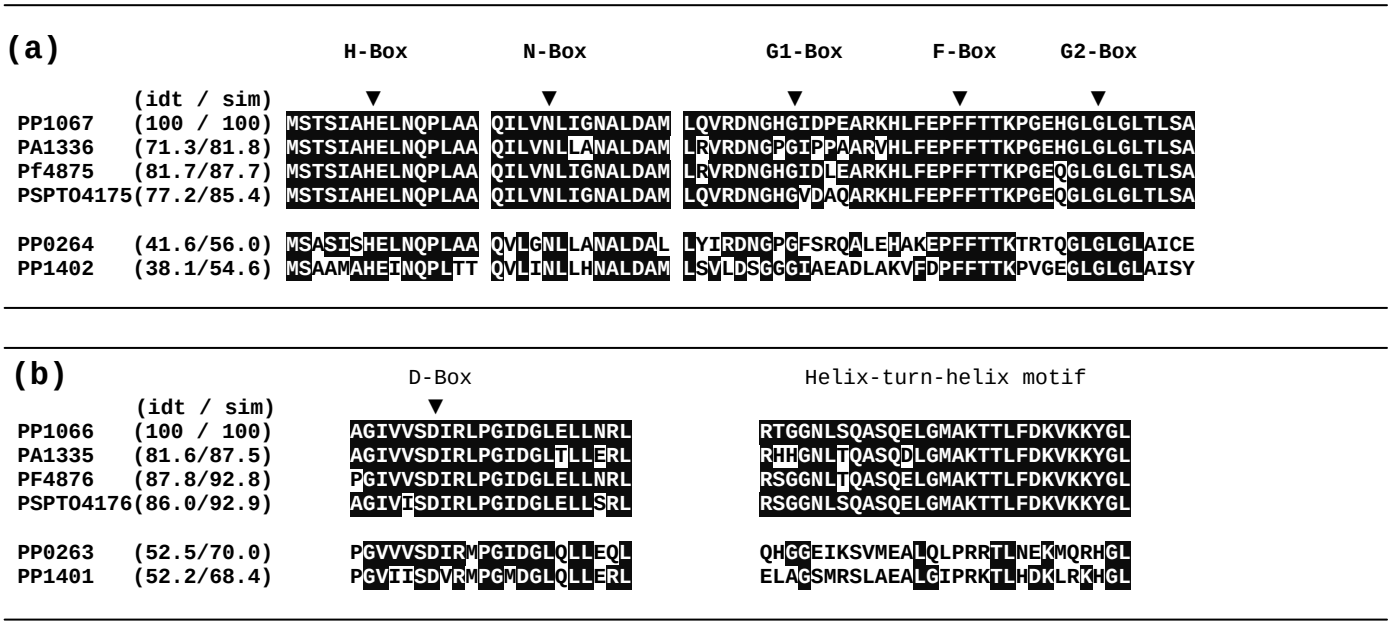


Figure 5.2: Partial sequence alignment of (Aau) Sensor kinase and response regulator in different species of Pseudomonas. **(a)** Sensor kinase with H-box, N-box, G1 and G2 boxes. **(b)** Response regulator with D-box; site of phosphate receiving from sensor kinase and helix-turn-helix DNA- binding motif. The abbreviation (idt / sim) are identities/similarities as calculated by Fasta3 alignment. The prefix of annotated genes are, PP- *P. putida*, PA- *P. aerugina* and PSPTO- *P. syringae*.

5.7 AatJMQP belongs to the family of polar amino acid ABC transporters

Besides the solute-binding protein, the transmembrane permease subunits also play an important role in the solute specificity and transport by ABC transporters. Broad-range solute ABC transporters typically have larger permeases (with 8-9 transmembrane domains) than permeases with specificity for a single substrate (5 transmembrane domains). The N-terminal segments of permease are exposed to the periplasmic space where they directly interact with solutes, and also play a role in stabilization of ligand-solute receptor complex (Kerppola *et al.* 1991).

An analysis of the N-terminal sequence of permeases AatM (PP1069) and AatQ (PP1070) showed distinct similarity with permeases of a sub-class of bacterial polar amino acid transporters first described by Walshaw *et al.* (Walshaw *et al.* 1997b). These transproters are widely distributed in Gram-negative microorganisms and exhibit a broad range of solute specificities. The N-terminal sequence alignment shown in Fig 5.3, indicates that certain key residues of the family are well

conserved in AatM and AatQ. These include a charged residue at position 30 (Arg or Lys), which seems to be required for stabilization of the solute since mutations at this position dramatically impair amino acid transport (Walshaw *et al.* 1997b). Another important residue, L 53, is thought to play a similar role in stability of solute binding and transport.

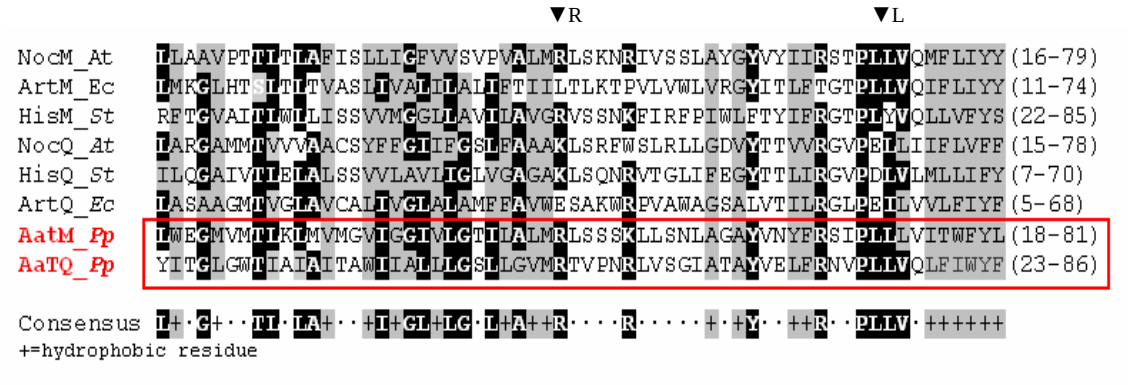


Figure 5.3: Partial sequence alignment of the N-terminal sequences of AatM (PP1070) and AatQ (PP1069) with other polar amino acid ABC transporters (Walshaw *et al.* 1997b). Functionally important residues are indicated by (▼) and transmembrane segments by gray background.

On the basis of sequence similarities, Tam and Saier have subdivided the group of bacterial extracellular solute-binding proteins (SBP) into 8 families, or clusters (Tam & Saier 1993). According to this system, AatJ belongs to SBP cluster 3 which groups together amino acid and opine-binding receptors. Blast similarity searches and alignments of AatJ with other proteins of this family showed that, at least 10 further bacterial solute receptors within SBP cluster 3 have marked selectivity for acidic amino acids (Glu and Asp). This suggestion, which originated from a homology model of the AatJ amino acid binding site, is supported by the amino acid binding properties of various AatJ mutants and by an analysis of the known SBP family 3 sequences. We also used the Pfam database (Finn *et al.* 2006) to perform a more extensive analysis of sequence similarities within SBP family 3 (Pfam entry SBP_bac_3). In the 78 sequences of the Pfam seed alignment, residue R93 was almost fully conserved (75 appearances), while R174 was conserved in 49 cases (6 sequences contained lysine at that position). Position 88 was occupied by threonine or serine in 49 cases. In position 71, a large aliphatic residue (L, I, M or V) was found in 53 of the 78 sequences. However, a distinct group of 13 proteins (17 % of the seed sequences) contained an arginine or lysine in position 71 (AatJ not included). Fig. 5.4 shows a partial alignment of the respective sequences with AatJ while Table 5.2 summarizes salient features of the proteins.

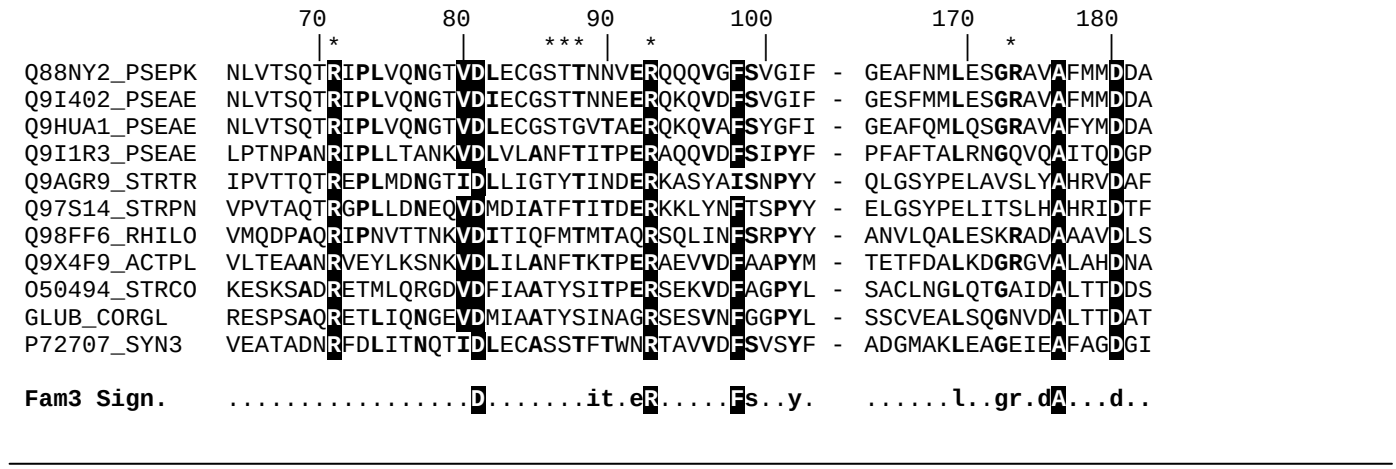


Figure 5.4: Partial sequence alignment of the putative Glu/Asp receptor sub-family identified in this alignment.

All solute binding proteins are monomeric and made up of two flexible lobes connected by a hinge. The active site is situated between these two lobes, which after ligand binding undergo a major conformational change with the result that both lobes bend inwards to trap the ligand. To date, crystal structures of three members of SBP cluster 3 proteins are available, i. e. GlnH from *E. coli* (PDB entries: 1ggg and 1wdn), HisJ from *Salmonella typhimurium* (1hpp) and the Arginine/lysine binding protein from the same organism (1laf, 1lag, 2lao). The modes of ligand binding to these proteins were compared in detail by Sun et al, (Sun *et al.* 1998). In all known SBP family 3 structures, the carboxylate group of the bound amino acid is stabilized by electrostatic interactions with an arginine residue that is strictly conserved in all family 3 proteins (in AatJ numbering, which will be used here and in the following, it is R93).

The homology model generated using GlnH as the template (cf. section 4.12 and Fig. 4.13) suggests a mode of ligand binding similar to those seen in the experimental structures, i. e. R93 and T88 (possibly S85 as well) interact with the α-carboxylate and α-ammonium group of the bound amino acid. Interestingly, in the modeled structure the γ-carboxylate of the ligand is positioned between two further arginine residues, R71 and R174, with R71 occupying the more favorable position. This result is clearly consistent with the observed preference of AatJ for acidic amino acids. In view of these data we predicted that the proteins listed in the Table 5.1 all act as

extracellular glutamate and/or aspartate receptors. In fact, besides AatJ only two members of the group (the GluB proteins of *Corynebacterium glutamicum* and *Streptomyces coelicolor*) have been correctly annotated as glutamate-binding proteins while the function of the others is unclear (the annotation of the *R. loti* mlr3796 gene product as glutamine-binding protein remains arguable).

Trembl Accession	**Organism**	**Gene**	**Annotated as**
Q88NY2_PSEPK	*P. putida* KT2440	*aatJ*	Periplasmic ligand-binding
Q9I1R3_PSEAE	*Pseudomonas aeruginosa*	PA2204	Probable ligand-binding
Q9I402_PSEAE	*Pseudomonas aeruginosa*	PA1342	Probable ligand-binding
Q9HUA1_PSEAE	*Pseudomonas aeruginosa*	PA5082	Probable ligand-binding
Q9AGR9_STRTR	*Streptococcus thermophilus*	*peb1*	Outer membrane bound
Q97S14_STRPN	*Streptococcus pneumoniae*	SP_0609	Probable ligand-binding
Q9X4F9_ACTPL	*Actinobacillus pleuropneumoniae*	*apaA*	Antigenic protein
GLUB_CORGL	*Corynebacterium glutamicum*	*gluB*	Glutamate-binding protein
O50494_STRCO	*Streptomyces coelicolor*	*gluB*	Glutamate-binding protein
P72707_SYN3	*Synechocystis sp.* PCC 6803	Sll0224	Outer membrane bound
Q98FF6_RHILO	*Rhizobium loti*	Mlr3796	Glutamine-binding protein
O83330_TREPA	*Treponema pallidum* *)	TP_0308	Probable ligand-binding
Q9RP92_STRPN	*Streptococcus pneumoniae* *)	*aatB*	Outer membrane bound
Q99ZD7_STRP1 K	*Streptococcus pyogenes* M1 *)	SPY_1274	ligand-binding protein

Table 5.1: Proteins belongs from a new subfamily of solute binding protein family 3. Proteins GluB of *C. glutamicum* and *S. coelicolor* are characterized as glutamate binding proteins. Asteric mark *) indicate the presence of lysine residue at position 71, rest of others have arginine at this position.

5.8 Aat is the only high-affinity glutamate transporter in *P. putida*

Only very few ABC transporters for glutamate uptake have been characterized in detail. Kronemeyer *et al.* (Kronemeyer *et al.* 1995) studied a glutamate importer of *Corynebacterium glutamicum* which showed close structural homology with other polar amino acid transporters. Another binding protein-dependent Glu/Asp ABC transporter GltIJKLM from *E. coli* was studied by (Linton & Higgins 1998). The sequence similarity between Glt and Aat of *P. putida* is around 70% (see Table 5.2). The *E. coli* Glt system was functionally characterized by Furlong and

coworkers (Willis *et al.* 1975 and Schellenberg & Furlong 1977). These workers estimated K_d values for amino acid binding to the solute-binding protein (GltI), reporting data (0.7 μM for Glu and 1.2 μM for Asp) are very similar to our K_d values for Glu and Asp binding to AatJ. In an another study with solute-binding protein (Peb1a) of an Asp/Glu-ABC transporter from *Campylobacter jejuni* (Thomas *et al.* 2006) K_d values of 0.5 μM for Asp and 0.79 μM for Glu were reported.

As judged by sequence similarity, the *P. putida* genome also encodes further transporters for Asp and/or Glu, i. e. proton-driven (GltP) and Na^+-driven (GltS) glutamate/aspartate transporters, which show 73% and 60% sequence similarity with GltP and GltS of *E. coli* (cf. Table 5.2). These transporters were studied in *E. coli* (Jacobs *et al.* 1995), yielding K_d values for Glu between 10-20 μM (Deguchi *et al.* 1989 and Tolner *et al.* 1995a). At present time, we have no experimental evidence indicating whether or not GltS and/or GltP are also expressed in *P. putida* KT2440 under normal conditions. However, we found, that increasing the external Na^+ concentration did not improve the growth of mutants KT*aauR*, KT*aatP* and KT*aatJ* on Glu as sole source of carbon and nitrogen (data not shown here). This indicates that the Na^+-dependent GltS system does not play a major role in Glu import by *P. putida* KT2440. There is also no evidence suggesting the presence of an additional low-affinity transporter besides Aat, since high concentrations of Glu (up to 10 mM) did not improve growth of KT*aatJ* or KT*aatP*.

5.9 Separate systems exist for Asp and/or Asn uptake

As shown in Fig. 4.2, Aat knock-out mutants KT*aatJ* and KT*aatP* grow well on aspartate or asparagine as sole source of carbon and nitrogen, indicating the presence of an alternate aspartate transporter in *P. putida* KT2440. While selective Asp transporters seem to be rare in microorganisms, many bacteria contain dicarboxylate importers (usually called Dct) that not only transport dicarboxylates but also transport Asp (Janausch *et al.* 2001).

Uptake system	*E. coli* K12	Identity (%)	Similarity (%)	*P. putida* KT2440	Function
ABC transporter (Glu/Asp)	GltI	55	69	AatJ (PP1071)	Solute-binding protein
	GltJ	49	68	AatM (PP1070)	Permease subunit
	GltK	52	66	AatQ (PP1069)	Permease subunit
	GltL	70	79	AatP (PP1068)	ATP-binding domain
Glu/Asp:H^+ symporter	GltP	62	73	GltP (PP0137)	Permease
Glu:Na^+ symporter	GltS	50	60	GltS (PP0996)	Permease
C_4-dicarboxylate/Asp:H^+ symporter	DctA	73	86	DctA (PP1188)	Permease

Table 5.2: Similarity of different putative/established Glu transporters of *E. coli* K12 and *P.putida* KT2440. The sequence similarities and identities were determined by Fasta3 alignment.

E. coli contains several Dct systems. The Dcu transporter, which is only expressed in anaerobic conditions, mediates dicarboxylate import for energy production via the so-called 'fumarate respiration' (Janausch *et al.* 2001). In aerobic conditions, many organisms use the DctA system, a binding protein-dependent transport system driven by an electrochemical proton gradient (Engelke *et al.* 1989 and Jording & Puhler 1993). A study by Reid *et al.* on Dct system of *Rhizobium leguminosarum* suggested that DctA is selectively induced when Asp or succinate was added as carbon and nitrogen source and are highly specific for aspartate uptake (Reid *et al.* 1996).

The DctA system of *P. putida* KT2440 is encoded by gene PP1188 (cf. Table 5.2) and is also present in many other *Pseudomonas* species. As shown by Fig. 5.5, *P. putida* DctA shows marked homology with well-characterized DctA transporters of *E. coli* and *R. leguminosarum* both of which are known to transport aspartate. We, therefore assume that growth of KT*aatP* and KT*aatJ* on Asp and Asn is mainly due to this transporter. Direct experimental evidence for the involvement of DctA is still unavailable.

5.10 The *aatPMQJ* operon is regulated by AauR

As already discussed above, the alternate sigma factor σ^{54} controls many genes related to nitrogen metabolism, among them *glnA, ntrB/ntrC,* and *ansB* (Eberl *et al.* 2000 and Sonawane *et al.* 2003b). The binding motif of AauR as identified by promoter deletion and DNase I foot printing experiments (cf. section 4.18) is present at -119 to -137 region of *aat* promoter related to transcription start. The core binding site for AauR (HTH motif) is **GTTCGG**NNNN**CCGAAC**, with inverted repeat of 6 bases within distance of 4 bases. This kind of HTH-binding motif is

common with DNA binding motif of HTH_FIS family of bacterial transcription regulators (Tzou & Hwang 1999). In addition, a typical -12/-24 motif (Barrios *et al.* 1999) was found in the *aat* promoter which is required for recruitment of the alternate sigma factor σ^{54} to the initial transcription complex.

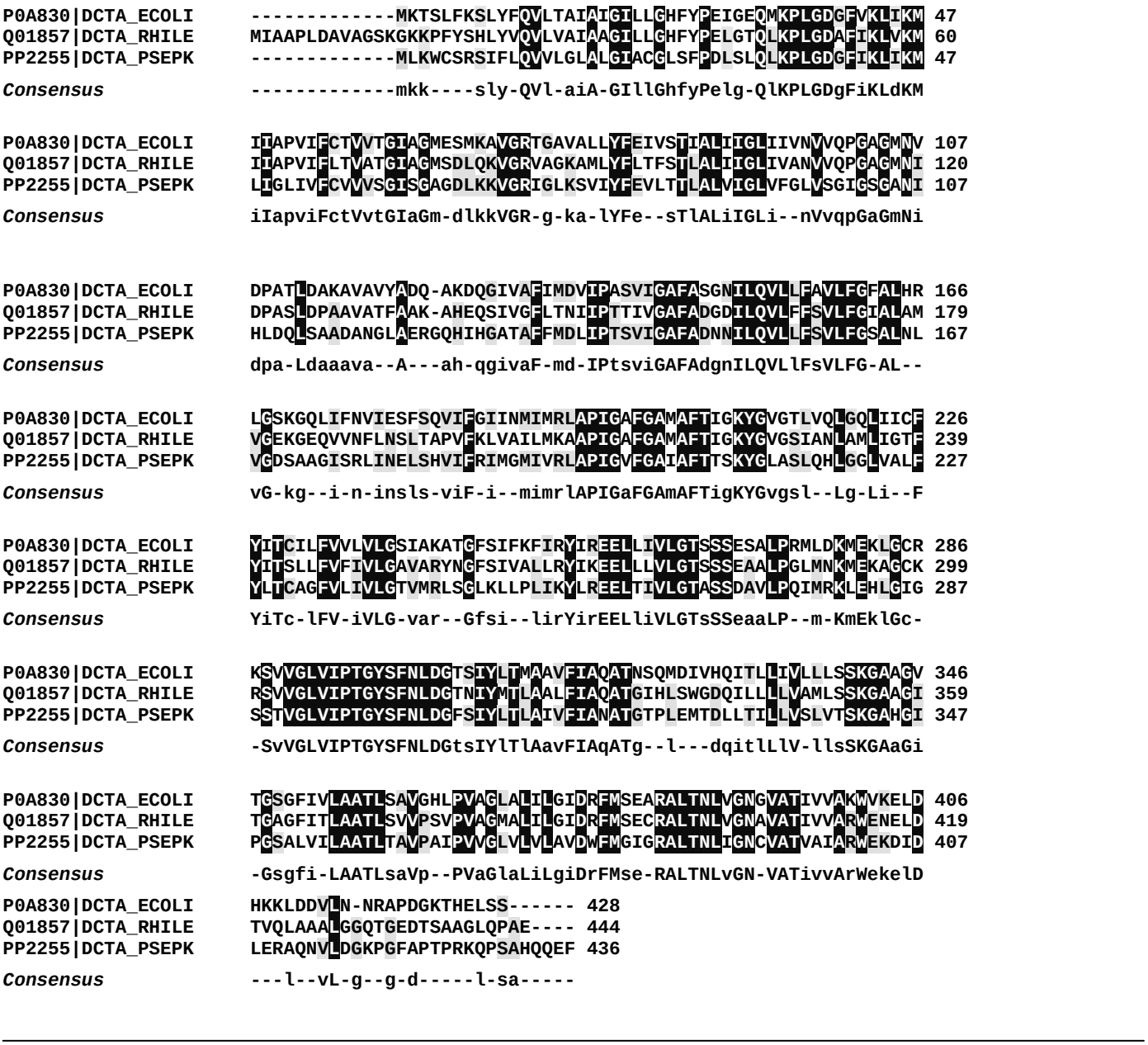


Figure 5.5: Alignment of the DctA proteins of *E. coli* (P0A830), *R. leguminosarum* (Q01875) and *P. putida* (PP2255). Highly conserved residues are shown white on black, while sequence similarities are highlighted by light grey background.

5.11 Further Aau-regulated genes in *P. putida*

In order to identify further AauR/S-responsive genes we screened the whole *P. putida* KT 2440 genome for the sequence CN**TTCGG**NNNT**CCGAA**N using the Fasta-Genome similarity search available online at http://www.ebi.ac.uk/fasta33/genomes.html. This query provided several clear hits which are summarized in Fig. 5.6. The respective genes and their known or putative functions are discussed in detail below.

Gene		Function/Annotation
PP1071	ATTGCTCGTTCGGTTATCCGAACGCA	Glu/Asp ABC transporter(*aat*)
PP0137	TGGTCTTGTTCGGGATTCCGAATGTT	Glu/Asp:H^+ symporter (*gltP*)
PP1141	CTTCGGTTTTCGGCTTGCCGAAGTGC	Branched-chain aa transporter(*bra*)
PP1469	GTGCGGCCTTCGGGTTACCGAACTGG	Thiol/disulfide exchange protein(*dsbC*)
PP2082	ATGATAAGTTCGGTTTGCCGAATGCG	Phosphoenolpyruvate synthase(*ppsA*)
PP2453	GCATTGCATTCGTAATTCCGAACAAA	Glutaminase/asparaginase motif 1(*ansB*)
PP2453	AAAAGGTGTTCGGTTTTTCGGAAAAG	Glutaminase/asparaginase motif 2(*ansB*)
Consensus	.......*g*TTCGGNNNNCCGAA*c*....	

Figure 5.6: Alignment of verified and putative AauR binding sites. The AauR binding motif within the *P. putida* KT2440 *aat* gene was experimentally identified in this work, all others were detected by *in silico* genome screening (see text).

5.11.1 Periplasmic glutaminase/asparaginase (PGA)

As expected, the AauR binding motif was detected in the promoter upstream region of the PGA-encoding *ansB* gene (PP2453) where it seems to occur twice. *Pseudomonas* glutaminase/ asparaginase (PGA), like other type II amidohydrolases, converts Gln and Asn to the respective dicarboxylates, which are then taken up into the cells (see 'Introduction' for details). In *E. coli*, the *ansB* gene product (called asparaginase II) is only expressed in anaerobic conditions and regulated by the *fnr* protein (Jerlstrom *et al.* 1989). Under anaerobiosis, the *E. coli* enzyme is probably required to convert asparagine to aspartate which is further degraded by aspartase to yield the energy substrate fumarate. In the strictly aerobic organism *P. putida*, which does not use this pathway, PGA is induced by its substrates (Gln, Asn) as well as by its products (Glu, Asp) and repressed by glucose and dicarboxylates. The negative mutants of *ansB* of *P. putida* KT2440 were defective in the utilization of Gln as sole source of carbon and nitrogen. (Sonawane *et al.* 2003a). Similar results were reported by Hüser et al, (Hüser *et al.* 1999) for *P. fluorescence* ATCC 13525.

These findings suggested the role of PGA in conversion of Gln to Glu for transport, because Gln is not transported by *P. putida.*

In the present work we found that KT*aauR* mutants are also defective in PGA activity showing a phenotype similar to that of *ansB* mutants (cf. section 4.6.1). This finding together with the identification of AauR-binding motifs in the *ansB* promoter strongly suggests that the AauR system indeed mediates the regulation of *ansB* by Glu and Asp.

5.11.2 Glu/Asp:H^+ transporter (GltP)

Another interesting hit in the search for AauR binding sites was the promoter upstream region of gene PP0137 which, by homology, encodes the secondary-active Glu transporter GltP (Fig 5.7). As already discussed above, this kind of symporter use a pH gradient as driving force for Glu import (de Vrij *et al.* 1989 and Jacobs *et al.* 1995). As shown by Fig. 5.7, GltP of *P. putida* KT2440 is closely similar to the GltPs of *E. coli* and *B. subtilis*. The function of *E. coli* GltP was studied in detail by Tolner *et al.* (Tolner *et al.* 1995b and Raunser *et al.* 2006). The activity of GltP might explain the fact that KT*aatP* mutants still grow on Glu, yet with prolonged lag phase and reduced cellular density (Fig 4.2). If GltP is indeed responsible for Glu import in these conditions, one has to assume, however, that glutamate transport by GltP requires the AatJ solute-binding protein, since mutant KT*aatJ* was totally unable to grow on glutamate. As already mentioned, the Glu/Na^+ symporter GltS (encoded by PP0996) is also present in *P. putida.* In view of the facts that i) this gene does not contain a AauR binding motif and ii) that addition of Na^+ did not improve growth of KT*aatJ* and KT*aauR* suggest that GltS of *P. putida* has no significant role in Glu uptake.

5.11.3 Branched chain amino acid transporter (Bra)

A highly conserved AauR binding motif was also detected in the promoter of the *bra* operon (PP1141-PP1137) which encodes the branched chain amino acid transporter BraCDEFDG. The branched chain amino acid transporter of *P. aeruginosa* was functionally characterized by Hoshino *et al.* (Hoshino *et al.* 1992) and by Hosie and coworkers (Hosie *et al.* 2001 and Hosie *et al.* 2002). In *R. leguminosarum* these amino acid transporters are bidirectional and also export amino acids in certain metabolic situations (Walshaw & Poole 1996 and Hosie *et al.* 2001). Homologous genes also exists in *E. coli* and in other *Pseudomonas spp.* with similar putative functions.

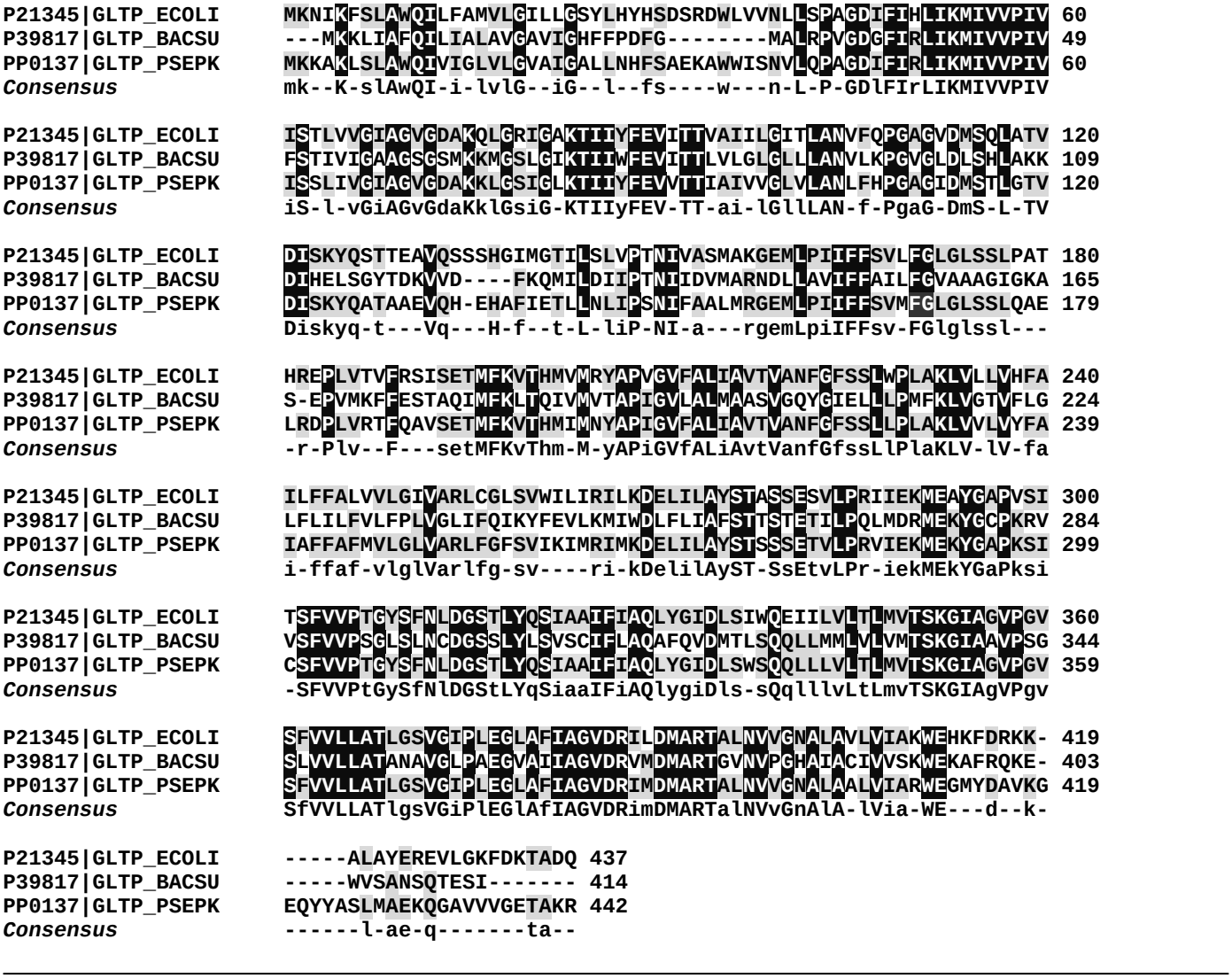


Figure 5.7: Alignment of GltP protein of *E. coli* (P21345), *B. subtilis* (P39817) and *P. putida* (PP0137). Highly conserved residues are shown white on black, while sequence similarities are highlighted by light grey background.

BraC of *P. putida* shows >40% sequence similarity to BraC of *R. leguminosarum* (see Fig 5.8). In *P. putida* KT2440 grown on Glu as sole source of carbon and nitrogen, expression of *bra* is downregulated (Sonawane, 2003c). Moreover, our experiments show that glutamate-grown KT*aauR* excretes Glu into the medium (cf. section 4.4), probably as a consequence of metabolic overflow as proposed for *R.* l*eguminosarum* (Hosie *et al.* 2001). In that case, binding of AauR to the *bra* promoter should inhibit expression of the operon (see below).

```
Q9L3M3|BraC_RHILV   -MKKSLLSAVALTAMLAFSGNAWADVLIAVAGPLTGPNAAFGAQLQKGAEQAAADINAAG 59
PP1141|BraC_PSEPK   MIKISKLFAAMVLAGVASHSFAADTIKIGIAGPKTGPVTQYGDMQFIGAKQAIKDINAAG 60
Consensus           --K-S-L-A----A--A----A-----I--AGP-TGP----G-----GA-QA--DINAAG

Q9L3M3|BraC_RHILV   GINGEQIKIELGDDVSDPKQGISVANKFAADGVKFVIGHFNSGVSIPASEVYAENGILRN 119
PP1141|BraC_PSEPK   GVDGKMLEAKEYDDACDPKQAVAVANKVVNDGVKFVVGHLCSSSTQPASDIYEDEGVIMI 120
Consensus           G--G--------DD--DPKQ---VANK---DGVKFV-GH--S----PAS--Y---G----

Q9L3M3|BraC_RHILV   HPGRDEPDLHGTGLWNTFRTCGRDDQQGAIAGKYLADHFKDAKIAVVHDKTPYGQGLADE 179
PP1141|BraC_PSEPK   TPAATSPEITARGYKLIFRTIGLDSAQGPAAGNYIADHVKPKVVAVLHDKQQYGEGIATA 180
Consensus           -P----P-----G----FRT-G-D--QG--AG-Y-ADH-K----AV-HDK--YG-G-A--

Q9L3M3|BraC_RHILV   TKKAMNAAGVTEVIYEGINVGDKDFSALIAKMKEAGVSIIYWGGLHTEAGLIIRQAADQG 239
PP1141|BraC_PSEPK   VKQTLESKGTKVAVFEGLNAGDKDFSSIIQKLKQNNVDFVYYGGYHPELGLILRQAQEKG 240
Consensus           -K------G------EG-N-GDKDFS--I-K-K---V---Y-GG-H-E-GLI-RQA---G

Q9L3M3|BraC_RHILV   LKATLVSGDGIVSNELASIAGDAVAGTLNTFGPDPTANPANKELVEKFKAAGFNP-EAYT 298
PP1141|BraC_PSEPK   LKAKFMGPEGVGNDSISQIAQNASEGLLVTLPKSFDADPANKKIVDAIKADGKDPSGPFV 300
Consensus           LKA------G--------IA--A--G-L-T------A-PANK--V---KA-G--P-----

Q9L3M3|BraC_RHILV   LYSYAAMQTIAGAAKAAGSLDPEAVAKAMKEKGPFPTVLGDISFDEKGDPKIPGYIMYEW 358
PP1141|BraC_PSEPK   FPAYSAVELIAQGIKKAGSDDTDKVAEAIH-KGTFKTPTGDLSFDDKGDLKDFKFVVYEW 359
Consensus           ---Y-A---IA---K-AGS-D---VA-A---KG-F-T--GD-SFD-KGD-K------YEW

Q9L3M3|BraC_RHILV   KKVRTASTATSRRHVSFAPLYGT 381
PP1141|BraC_PSEPK   HFGKPKTEVSPQ----------- 371
Consensus           ----------------------
```

Figure 5.8: Alignment of the BraC proteins of *R. leguminosarum* (Q9L3M3) and *P. Putida* KT2440 (PP1441). Conserved residues are shown white on black background.

5.11.4 Thiol/disulphide exchange protein (DsbC)

DsbC belongs to the thiol-disulphide reductase family of proteins. It functions as a chaperon, assisting disulphide bond formation in periplasmic proteins of Gram-negative bacteria. In this repect it plays an important role in the type II secretion pathway. The Dsb family of *E. coli* has been studied in detail (Sone *et al.* 1997 and Khyse-Andersen 1984). In pseudomonads many proteins are secreted by type II pathway, involving about 12 different Xcp proteins for functional protein secretion (Filloux *et al.* 1998). Stenson and coworkers (Stenson & Weiss 2002) showed that the function of a toxin of *Bordetella pertussis* was seriously impaired in DsbA and DsbC mutants. The DipZ protein of *P. aeruginosa*, also a member of the Dsb family, was characterized by Page *et al.*, (Page *et al.* 1997). It encodes a disulphide reductase and plays an important role in the folding of c-type cytochromes. A study by Urban *et al.* on DsbA and DsbC of *P. aeruginosa*

showed that these proteins are involved in the folding of periplasmic enzymes like alkaline phosphatase, elastase and lipases (Urban *et al.* 2001).

The DsbC protein of *P. putida* KT2440 is clearly related to DsbC of *P. aeruginosa* (69% similarity) and DsbC of *E. coli* (32%) with several fully conserved motifs (Fig. 5.9). Although experimental data are presently unavailable, we speculate that DsbC of *P. putida* KT2440 may play a role in the folding of periplasmic proteins related to Glu uptake like PGA and AatJ.

```
P21892|DSBC_ECO57     --------------MKKGFMLFTLLAAFSGFAQADD--------AATQQTLAKMGIKS-- 36
PA3737|DSBC_PSEAE     -------------MRVTRFLAAAALGLMSTLALADN------ADQNIRKTLQALQPDLPI 41
PP1469|DSBC_PSEPK     MAPSGPCPPSGVPMRVTQFFAAAALALASTFAVAAATDSNAGAEQAIRKSLQNLELEVPV 60
Consensus             --------------rvt-F-aaaaLal-StfA-A--------a-qaIrk-Lq-l----p-

P21892|DSBC_ECO57     SDIQPAPVAGMKTVLTNSG-VLYITDDGKHIIQGPMYDVSGTAPVNVTNKMLLKQLNALE 95
PA3737|DSBC_PSEAE     DSIASSPLQGLYQVQLKGGRVLYASADGQFVMQGYLYQVKDGKPVNLTEKAESQAIAKAI 101
PP1469|DSBC_PSEPK     ESVASSPLNGLYEVKLQGGRVLYASADGQFVMQGYLFQIQDGKPVNLTEKTERQGIAKLI 120
Consensus             -siassPl-Gly-V---gGrVLYasaDGqfvmQGyl-q--dgkPVNlTeK-e-q-iakli

P21892|DSBC_ECO57     K-----EMIVYKAP-QEKHVITVFTDITCGYCHKLHEQMADYNALGITVRYLAFPRQGLD 149
PA3737|DSBC_PSEAE     NGVPASEMVVYPAKGQAKAHITVFTDTTCPYCQKLHAEVPDLTEQGIEVRYMAFPRQGPQ 161
PP1469|DSBC_PSEPK     NGIPAGEMVVYPAKGETKSHITVFTDTTCPYCHKLHAEVPELNRRGIEVRYVAFPRQGLG 180
Consensus             ng-pa-EmvVYpAkg--K-hITVFTDtTCpYChKLHaevp-ln--GIeVRY-AFPROGl-

P21892|DSBC_ECO57     SDAEKEMKAIWCAKDKNKAFDDVMAGKSVAPASCDVDIADHYALGVQLGVSGTPAVVLSN 209
PA3737|DSBC_PSEAE     SAGDKQLQAVWCAKEPTKAMDAMMNGKEIKSSECKNPVDKQFQMGQMVGVQGTPAIVLAN 221
PP1469|DSBC_PSEPK     SSGDQQLQAVWCSSDRRGAMDKMVEGEEIKAAKCSNPVSKQFQLGQSIGVNGTPAIVLES 240
Consensus             S-gd-qlqAvWC--d---Amd-m--G-eik-a-Q-npv-kqfqlGq--GV-GTPAiVL--

P21892|DSBC_ECO57     GTLVPGYQPPKEMKEFLDEHQKMTSGK 236
PA3737|DSBC_PSEAE     GQLLPGYQPAKQLAKLALEAK------ 242
PP1469|DSBC_PSEPK     GQVIPGYQPAPQVAKLALAK------- 260
Consensus             Gq--PGYQPa-q-aklal---------
```

Figure 5.9: Alignment of the DsbC proteins of *E. coli* (P21892), *P. aeruginosa* (PA3737) and *P. putida* KT2440 (PP1469). Highly conserved residues are shown white on black, while sequence similarities are highlighted by light grey background.

5.11.5 Phosphoenolpyruvate synthase (*PpsA*)

Phosphoenolpyruvate synthase (PpsA) is a key enzyme of gluconeogenesis in prokaryotes, where it revert the pyruvate kinase reaction by converting pyruvate to phosphoenolpyruvate (Niersbach *et al.* 1992). It is especially important when lactate and pyruvate are the only pecursors for gluconeogenesis, as *ppsA* mutants of *E. coli* did not grow in pyruvate and lactate as sole sources of carbon. Over-expression of *pcK* (PEP carboxykinase) or *ppsA* in *E. coli* accelerated growth several

fold when succinate or pyruvate were used as carbon source (Chao *et al.* 1993). In *E. coli* the *ppsA* gene is controlled by the global regulator FruR (Ramseier *et al.* 1995 and Negre *et al.* 1998). In *Pseudomonas* FruR functions as repressor of rather than as pleiotropic regulator of carbon flux through metabolic pathways (Reizer *et al.* 1999 and Duque *et al.* 2007). The control of *ppsA* expression in *P. putida* by AauR may help to provide glucose or intermediates of gluconeogenesis when the cells are grown in Glu as sole source of carbon and nitrogen.

5.12 Acidic amino acid utilization by *P. putida*: The current picture

The results described here together with published data from our group allow us to outline a consistent, although incomplete, picture of acidic amino acid uptake and utilization by *P. putida* KT2440 with emphasis on AauR as a key regulator. The AauR/S system exerts direct effects on the expression of a number of genes involved in amino acid import and metabolization, which are accompanied by indirect pleiotropic effects on the activities of other enzymes of nitrogen metabolism. The role of AauR in genetic control mechanisms is shown in Fig. 5.10 while important enzymes of Asp and Glu degradation are summarized in Fig. 5.11.

The acidic amino acids and their amides can pass through outer membrane porins (Sonawane & Rohm 2004) and become available in the periplasmic space. Here, Gln and Asn are converted to glutamate and aspartate by PGA. The interaction of Glu/Asp with the sensing domain of AauS induces autophosphorylation at its kinase domain. The phosphorylated AauS then transfers the phosphate to AauR which is activated and controls the expression of two groups of genes.

i) It activates the transcription *aat* and *gltP* (Glu-Asp/H+ symporter) and inhibits expression of *bra*, where Aat and GltP control Glu import, while BraC regulates Glu export under conditions of metabolic overflow.

ii) It activates the expression of enzymes directly or indirectly involved in amino acid metabolism, among them PGA which hydrolyzes Gln and Asn to allow high-affinity uptake of the hydrolysis products, DsbC which might be needed for proper folding of periplasmic proteins, and pyruvate synthase (PpsA) which channels the carbon skeleton of Asp and Glu into gluconeogenesis.

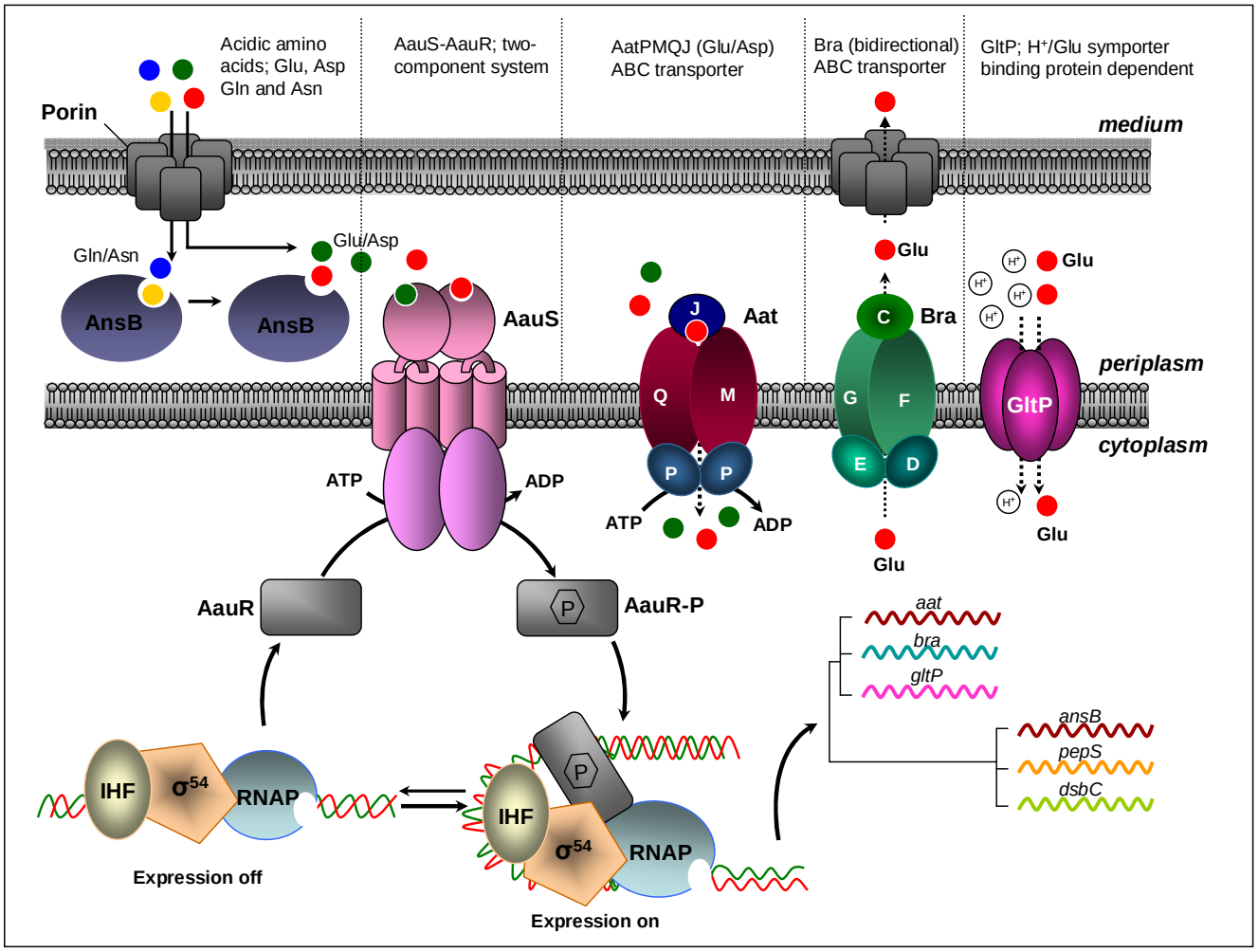


Figure 5.10: Proposed scheme of AauR-AauS two-component system regulation of acidic amino acid metabolism and genetics in *Pseudomonas putida*.

Gluconeogenesis is crucial for the synthesis of glucose and intermediates of glycolysis when amino acids are the sole sources of carbon. As shown in Fig. 5.11, pyruvate synthase (PpsA, enzyme 8) has a key position in this process as it reverts the pyruvate kinase reaction generating phosphoenol pyruvate (PEP) from pyruvate. Pyruvate can be generated from Asp and Glu in a number of ways. Both amino acids can be inter-converted by aspartate transaminase (reaction 2) or desaminated to TCA cycle intermediates (oxaloacetate, α-ketoglutarate or fumarate, reactions 3-5). Both oxaloacetate and malate are then converted to pyruvate, the substrate of PpsA (reactions 6-7).

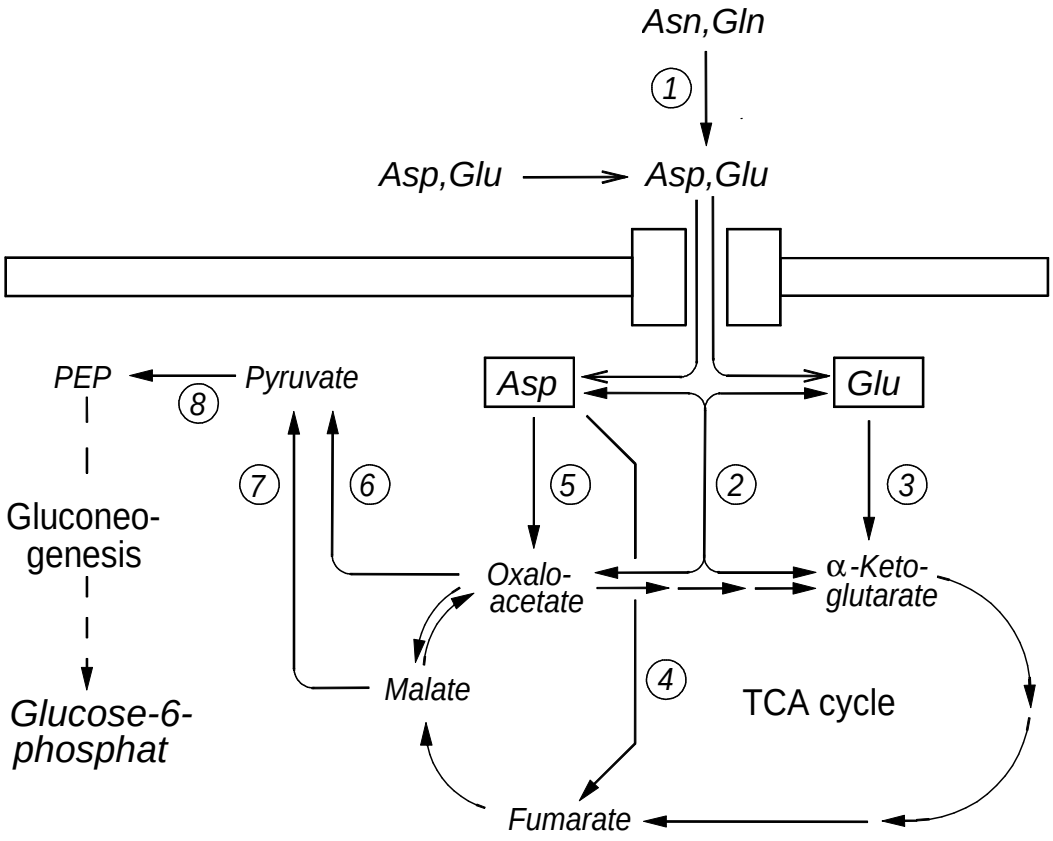


Figure 5.11: Proposed scheme of acidic amino acid utilization in *P. putida* KT2440. The shown are, 1- periplasmic glutaminase/asparaginase (PGA), 2 - aspartate transaminase, 3 – glutamate dehydrogenase, 4- aspartate lyase (aspartase), 5 - aspartate oxidase, 6 - oxaloacetate decarboxylase, 7 - malic enzyme, 8 - PEP synthase (PpsA). For details, see text.

Of course, many details of this scheme are still speculative at present time. So, for instance the involvement of GltP in glutamate uptake and its regulation by AauR have to be substantiated by experimental evidence. Moreover, the transport system (or systems) for aspartate uptake should be identified. Finally, the proposed key role of PpsA for growth of *P. putida* on acidic amino acids must be supported by experimental data. These and other experiments could not be completed in the course of the present study.

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7. Summary

The Gram-negative soil bacterium *Pseudomonad putida* is able to utilize an unusually large number of organic compounds as sources of energy and biomass, among them the acidic amino acids glutamate (Glu) and aspartate (Asp) and their amides glutamine (Gln) and asparagine (Asn). During growth of *P. putida* on any of these amino acids, the expression of a defined set of genes is induced that allow for their utilization. As reported previously, the Aau two-component system is involved in this adaptation process.

In the present study we analyzed the functional role of the AauR-AauS system in the uptake and metabolism of acidic amino acid by *P. putida* KT2440. *Aau*-negative mutants were defective in growth Glu and Asp as sole sources of carbon and nitrogen as well as in Glu and Asp uptake. In addition, the AauR mutant failed to express periplasmic glutaminase/ asparaginase (PGA), an enzyme required for the conversion of Gln and Asn to the respective acidic amino acids. Other enzymes involved in the assimilation of Glu and Asp also showed marked changes in activity. A*auR* deletion mutants grown on Asn and Asp started to accumulate Glu in the late log phase to levels many times higher than in the wild type and also excreted Glu if glucose was available. The excretion of glutamate in these conditions is probably mediated by Bra (a bi-directional ABC transporter), as reported for TCA cycle mutants of other rhizobacteria. The glutamate accumulation by *aau* mutants may be due to high aspartase activities which feed the carbon skeletons of Asp and Asn into the TCA cycle where glutamate dehydrogenase converts it to glutamate.

Immediately upstream of the *aau* locus of *P. putida* KT2440, an operon involving genes PP1068-PP1071 encodes an ABC transporter which we named Aat (for **a**cidic **a**mino acid **t**ransporter). Aat is also upregulated when *P. putida* is grown on acidic amino acids or their amides. AatPMQJ is a polar amino acid transporter belonging to the solute-binding protein family (SBP_Bac_3). The Aat system consists of four subunits, where AatP is the nucleotide binding protein, AatM and Aat Q are membrane spanning permeases and AatJ is the periplasmic solute-binding protein. The expressed and purified solute binding protein (AatJ) showed high binding affinity towards Glu and Asp (K_d = 0.4 μM and 1.3 μM respectively), while Gln and Asn as well as other dicarboxylates were bound with much lower affinity. A modeled structure of AatJ (using the glutamine- binding protein GlnH of *E. coli* as template) suggested a novel arrangement of active site residues which include several arginine residues. The modeled structure was validated by site

directed mutagenesis of several AatJ residues predicted to be in contact with the bound ligand. A data base search suggested that AatJ and at least 15 further proteins constitute a new subfamily of periplasmic acidic amino acid receptors.

The role of the AauRS two-component system in the transcriptional regulation of *aat* and the PGA-encoding *ansB* gene was analyzed by gel-shift assays. The purified recombinant protein AauR was shown to bind to the promoter regions of both *aat* and *ansB*. A DNase I footprinting analysis revealed that the AauR binding motif consists of a well-conserved inverted repeat of six nucleotides (**GTTCGG**NNNN**CCGAAC**). By *in-silico* analysis of the *P. putida* KT2440 genome, several other genes were detected that contain an AauR interaction motif in their promoters. These genes encode a H^+/Glu symporter (GltP), phosphoenolpyruvate synthase (PpsA), a branched-chain amino acid transporter (Bra) and a disulphide exchange protein (DsbC). Based on these data, we give an outline of acidic amino acid uptake and metabolism in *P. putida* and its regulation by the AauRS two-component system.

7. Zusammenfassung

Das Gram-negative Bodenbakterium *Pseudomonas putida* ist in der Lage, eine ungewöhnlich große Zahl organischer Verbindungen als Quelle für Energie und Biomasse zu nutzen. Unter diesen Verbindungen sind auch die sauren Aminosäuren Glutamat (Glu) und Aspartat (Asp) sowie deren Amide Glutamin (Gln) und Asparagin (Asn). Wächst *P. putida* auf einer dieser Verbindungen, wird eine Gruppe von Genen induziert, die für Aufnahme und Verwertung dieser Aminosäuren notwendig sind. Aus früheren Untersuchungen war bereits bekannt, dass das Zweikomponentensystem AauRS an diesem Anpassungsprozess beteiligt ist.

In der vorliegenden Arbeit untersuchten wir am Beispiel des Stammes *P. putida* KT2440 die Rolle des Aau-Systems bei der Aufnahme und Verstoffwechslung der sauren Aminosäuren im Einzelnen. *Aau*-negative Mutanten waren unfähig, auf Glu and Gln als einziger C- und N-Quelle zu wachsen und zeigten auch massive Defekte in der Aufnahme von Glu und Asp. Außerdem waren sie nicht mehr in der Lage, periplasmatische Glutaminase/Asparaginase (PGA) zu exprimieren, ein Enzym das die die Hydrolyse von Gln und Asn zu den betreffenden Dicarboxylaten katalysiert. Auch andere Enzyme des Glu- und Asp-Stoffwechsel waren in ihrer Aktivität verändert. Mutanten mit inaktiviertem AauR akkumulierten beim Wachstums auf Asn und Asp intrazellulär große Mengen von Glutamat. Die dabei auftretenden Glutamatspiegel waren um ein Vielfaches höher als in Wildtyp-Zellen. Diese Exkretion von Glutamat, die auch bei Citratzyklus-Mutanten anderer Rhizobakterien beobachtet wurde, wird wahrscheinlich durch den bidrektionalen ABC-Transporter Bra bewirkt. Die Anhäufung von Glutamat geht vermutlich auf die in AauR-Mutanten erhöhten Aspartase-Aktivitäten zurück. Dieses Enzym kanalisiert das Kohlenstoffskelett von Asp in den Citratzyklus, von wo aus durch die Glutamatdehydrogenase Glutamat gebildet werden kann.

Im Genom von *P. putida* KT2440 liegt direkt neben *aau* ein weiteres Operon, das von den Genen PP1068-PP1071 gebildet wird und für einen ABC-Transporter kodiert. Wir haben diesen Transporter Aat benannt (für **a**cidic **a**mino acid **t**ransporter). Aat wird hochreguliert, wenn *P. putida* auf sauren Aminosäuren oder deren Amiden wächst. Das AatPMQJ-System ist ein Transporter für polare Aminosäuren und gehört zur Gruppe 3 der Familie der periplasmatischen Ligandenbindungsproteine (SBP_Bac_3). Der Aat-Transporter besteht aus vier Untereinheiten,

dem Nucleotid bindenden Protein AatP, zwei die Membran durchspannenden Permease-Einheiten (AatM und Aat Q) sowie dem periplasmatischen Bindeprotein AatJ. Rekombinant exprimiertes und gereinigtes AatJ zeigte hohe Affinität zu Glu und Asp (K_d = 0.4 μM bzw. 1.3 μM), während Gln und Asn und andere Dicarboxylate mit viel geringerer Affinität gebunden werden. Eine (auf Grundlge des Glutamin bindenden Proteins GlnH von *E. coli*) durch Homologiemodelling erzeugte AatJ-Struktur legte nahe, dass sich sein Ligandenbindungszentrum von dem anderer Bindeproteine unterscheidet, wobei mehrere Argininreste maßgeblich sind. Die modellierte Struktur wurde experimentell bestätigt, indem diverse Aminosäurereste, die nach dem Modell an Wechselwirkungen mit dem Liganden beteiligt sein sollten, durch gerichtete Mutagenese verändert wurden. Eine Suche in Sequenzdatenbanken zeigte, dass AatJ zusammen mit mindestens 15 weiteren Proteinen eine neue Subfamilie der periplasmatischen Bindeproteine bildet.

Die Beteiligung des Zweikomponentensystems AauRS an der Regulation der Transkription von *aat* und *ansB* (dem für PGA kodierenden Gen) wurde mit Hilfe des EMSA (electrophoretic mobility shift assay) nachgewiesen. Dabei zeigte sich, dass gereinigtes AauR an beide Promotoren bindet. Die genaue Bindungsstelle wurde durch DNAaseI-„Footprints“ identifiziert. Sie besteht in einem hoch konservierten Inverted Repeat aus je 6 bp, die durch 4 bp getrennt sind (**GTTCGG**NNNN**CCGAAC**). Bei einer *in-silico* Suche im *P. putida* KT2440-Genom wurden zusätzliche Gene entdeckt, die ebenfalls das AauR-Bindemotiv in ihrer Promoterregion enthalten. Zu den Produkten dieser Gene gehören ein H^+/Glu-Symporter (GltP), das Enzym Phosphoenolpyruvat-Synthase (PpsA), ein ABC-Transporter für verzweigtkettige Aminosäuren (Bra) and das Disulfidaustauschprotein DsbC. Auf der Grundlage dieser Ergebnisse war es erstmals möglich, am Beispiel *P. putida* KT2440 konkrete Vorstellungen zur Aufnahme und Metabolisierung der sauren Aminosäuren und ihrer Regulation durch das AauRS Zweikomponentensystem zu entwickeln.

8. Appendix

8.1 Abbreviations

8.1.1 Amino acids

Ala (A)	**A**lanine
Arg (R)	**A**rginine
Asn (N)	**A**sparagine
Asp (D)	**A**spartate
Cys (C)	**C**ysteine
Gln (Q)	**G**lutamine
Glu (E)	**G**lutamine
Gly (G)	**G**lycine
His (H)	**H**istidine
Ile (I)	**I**soleucine
Leu (L)	**L**eucine
Lys (K)	**L**ysine
Met (M)	**M**ethionine
Phe (F)	**P**henylalanine
Pro (P)	**P**roline
Ser (S)	**S**erine
Thr (T)	**T**hreonine
Tyr (Y)	**T**yrosine
Val (V)	**V**aline

8.1.2 Antibiotics

Amp	**A**mpicillin
Cb	**C**arbenicillin
Cmp	**C**hloramphenicol
Gm	**G**entamycin
Kan	**K**anamycin
Tet	**T**etracycline

8.1.3 Enzymes

AR	**A**denylyl **r**emoving
ATase	**A**denylyl**t**ransferase
CIP	**C**alf **i**ntestinal alkaline **p**hosphatase
GDH	**G**lutamate **d**ehydrogenase
GOGAT	**G**lutamate **s**ynthase
GS	**G**lutamine **s**ynthetase
HPK	**H**istidine **p**rotein **k**inase
PGA	**P**eriplasmic **g**lutaminase/**a**sparaginase
UR	**U**ridylyl **r**emoving
UT	**U**ridylyl**t**ransferase

8.1.4 Microorganisms

A. brasilense	*Azospirillum brasilense*
B. subtilis	*Bacillus subtilis*
C. glutamicum	*Corynebacterium glutamicum*
C. Jejuni	*Campylobacter jejuni*
E. chrysanthemi	*Erwinia chrysanthemi*
E. coli	*Escherichia coli*
P. putida (PP)	*Klebsiella pneumoniae*
P. aeruginosa	*Pseudomonas aeruginosa*
P. syringae	*Pseudomonas syringae*
P. fluorescens	*Pseudomonas fluorescens*
R. meliloti	*Rhizobium meliloti*
S. aureus	*Staphylococcus aureus*
R. leguminosarum	*Rhizobium leguminosarum*
S. meliloti	*Sinorhizobium meliloti*
S. typhimurium	*Salmonella typhimurium*

8.1.5 Genes/Proteins

ansB	Periplasmic glutaminase/asparaginase gene
Bra	Branched chain amino acid ABC-transporter
dct	**D**i**c**arboxylic **t**ransport system
aau	**A**cidic **a**mino acid **u**tilization
aauR	Gene encoding response regulator AauR
aauS	Gene encoding sensor kinase AauS
aat	**A**cidic **a**mino acid **t**ransporter
aatP	Glutamate receptor encoding gene
aatJ	ATP binding protein encoding gene
aatM/aatQ	Aat permeases genes
dctD	Dicarboxylate transport regulator gene
gltB	Major subunit of glutamate synthase Gene
HisJ	Histidine binding protein
Ntr	**N**itrogen **r**egulatory system
Peb1a	Periplasmic Glu/Asp binding protein of *C. Jejuni*
ppsA	Phosphoenolpyruvate synthase gene

8.1.6 General

Abbreviation	**Full name**
α-KG	**A**lpha-**k**eto**g**lutarate
A	**A**bsorption

Å	**A**ngstrom [10^{-8} cm]
ABC	**A**TP **b**inding **c**assette
ADP	**A**denine **di**ph**o**sphate
AMP	**A**denine **m**ono**p**hosphate
APS	**A**mmonium **p**ersulfate
ATP	**A**denine **t**riphosphate
bp	**B**ase **p**air
cAMP	**C**yclic **a**denine **m**ono**p**hosphate
CCR	**C**arbon **c**atabolite **r**epression
cDNA	**C**omplementary **D**NA
CIP	**C**alf **i**ntestinal **p**hosphatase
Da	**D**alton
DNA	**D**eoxy**n**ucleic **A**cid
DTT	**D**i**t**hio**t**hreitol
EDTA	**E**thylene**d**iamine**t**etracetic **a**cid
g	**R**elative centrifugal force
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography
HEPES	4-(2-**h**ydroxy**e**thyl)**p**iperazine-1-**e**thane**s**ulphonic acid
IPTG	**I**so**p**ropyl β-D **t**hio**g**alactoside
IR	**I**nverted repeats
kb	**K**ilo**b**ase
kDa	**K**ilo**d**alton
L-AHA	**L**-**a**spartic acid β-**h**ydroxamate
LB	**L**uria-**B**ertani medium
M	**M**olar
mg	**m**ili**g**ram
min	**M**inute
N	**N**itrogen source
nt	**N**ucleo**t**ide
OAA	**O**xalo**a**cetic **a**cid
^{o}C	**D**egree **c**entigrade
OD	**O**ptical **d**ensity
ORF	**O**pen **r**eading **f**rame
PCR	**P**olymerase **c**hain **r**eaction
PGPR	**P**lant **g**rowth **p**romoting **r**hizobacteria
Pi	**I**norganic **p**hosphate
pI	**I**soelectric **p**oint
RNA	**R**ibo**n**ucleic **a**cid
rpm	**R**evolutions **p**er **m**inute
RR	**R**esponse **r**egulator
SBP	**S**olute **b**inding **p**rotein
SDS	**S**odium **d**odecyl **s**ulfate
TBE	**T**ris **b**orate-**E**DTA buffer
TCA	**T**ri**c**arboxylic **a**cid

TE	**T**ris-**E**DTA buffer
TEMED	**N' N' N' N'-Tetramethylendiamine**
Tris	Tris(hydroxymethyl)-aminomethane
U	Activity unit ($\mu mol.min^{-1}$)
UTP	**U**ridine **t**ri**p**hosphate
UV	**U**ltra **v**oilet
V	**V**olt
v/v	**V**olume by **v**olume
W	**W**att
w/v	**W**eight by **v**olume
WT	**W**ild **t**ype

8.2 Curriculum Vitae

Name: SINGH, Birendra

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Educational Qualifications:

Primary Education:

1982-1990 Primary and Junior Highschool, U. P. Govt. India

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Jan 2005-Jan 2008 Institute for Physiological Chemistry**,** Philipps University Marburg (Molecular Enzymology Group, Prof. Röhm) "Functional and biochemical analysis of acidic amino acid transport and utilization by *Pseudomonas putida* KT2440".

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8.4 Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin zur Promotionsprüfung eingereichte Arbeit mit dem Titel “Functional and biochemical analysis of acidic amino acid transport and utilization by *Pseudomonas putida* KT2440” im Institut für Physiologische Chemie, Arbeitsgruppe Molekulare Enzymologie unter der Leitung von Herrn Prof. Dr. Röhm ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angegebenen Hilfmittel benutzt habe. Ein Teil der Experimente wurde unter Anleitung von Herrn Prof. Dr. Jörg Kämper, Max Planck Institut für Terrestrische Mikrobiologie, Marburg durchgeführt.

Ich habe bisher an keinem in- und ausländischen medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Teile der vorliegenden Arbeit wurden bereits wie folgt veröffentlich:

Originalarbeiten

Singh Birendra and Röhm KH. 2008. Characterization of a *Pseudomonad putida* ABC transporter (Aat) required for acidic amino acid uptake: Biochemical properties and regulation by the Aau two-component system. Microbiology, *In Press.*

Singh Birendra and Röhm KH. 2008. A new subfamily of bacterial glutamate/aspartate receptors. Biological Chemistry. 389(1): 33-36.

Sonawane A., **Singh Birendra** and Röhm KH. 2006. The AauR-AauS two-component system regulates uptake and metabolism of acidic amino acids in *Pseudomonas putida*. Applied and Environmental Microbiology. 72(10):6569-77.

Posterpräsentationen

Birendra Singh, Avinash Sonawane and KH Rohm. Characterization of novel glutamate/ aspartate ABC transporters in *Pseudomonads*. Society of General and Applied Microbiology (VAAM conference), Osnabrucke, Germany, 1-4th April 2007.

Birendra Singh, Avinash Sonawane and KH Rohm. Role of the *aau* two-component system in *Pseudomonas putida* KT2440. Pseudomonas 2005 Conference, Palais des Congres-Marseille, France, 28-31st August, 2005.

Marburg, den 12. October 2007 Birendra Singh